AD_____

Award Number: DAMD17-96-1-6324

TITLE: A Novel Myc-Interacting Protein with Features of a Breast Tumor Suppressor Gene Product

PRINCIPAL INVESTIGATOR: Frank K. Rauscher, Ph.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, Pennsylvania 19104-4268

REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (15 Sep 96 - 14 Sep 00)

4. TITLE AND SUBTITLE
A Novel Myc-Interacting Protein with Features of a Breast Tumor Suppressor Gene Product

5. FUNDING NUMBERS
DAMD17-96-1-6324

6. AUTHOR(S)
Frank K. Rauscher, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
Philadelphia, Pennsylvania 19104-4268

E-MAIL:
rauscher@wistar.upenn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains color photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
110

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Foreword

This research project was founded on our discovery of the Bin1 gene, which encodes a novel adaptor protein (originally known as '99') that has tumor suppressor properties in breast cancer. Bin1 interacts with Myc, an oncoprotein widely activated in breast cancer, and mechanistic investigations in our laboratory suggests that Bin1 has a role in mediating apoptosis by Myc under certain conditions.

This project exceeded its planned aims, including the following milestones. Characterization of the Bin1 gene and its multiple gene products allowed an intensive analysis of Bin1 structure and expression in 50 breast cancer cases and multiple breast cancer cell lines. We found evidence of >50% losses in primary breast cancer, mainly due to gene silencing. Preliminary evidence from a second study suggest that losses are correlated with an increased incidence of lymph node metastases. Cell biological analyses defined universal losses of Bin1 in malignant cell lines. Re-expression of Bin1 in such settings engaged a programmed cell death process that resembled apoptosis. This process was independent of p53, Rb, Bcl-2 family proteins, and caspases, suggesting that Bin1 participates in a unique cell death mechanism. A functional analysis of Bin1 identified crucial effector regions, laying the foundation for current work to identify effector proteins. Effector candidates include Ku proteins, which are involved in the DNA damage response, and Bin3, a Bin1-related partner protein that localizes to mitochondria in cells. As part of this work we also cloned Bin2, a Bin1-related partner protein with non-cancer-related functions in hematopoietic cells. Finally, capping previous work, we cloned and characterized the mouse Bin1 gene and identified a function for it in muscle cell differentiation.

The mouse gene cloning enabled the generation of a Bin1 "knockout" mouse, the characterization of which has been the focus of work this year. Loss of Bin1 leads to embryonic lethality, probably due to cardiac muscle hypertrophy. Interestingly, embryos exhibit no evidence of gross defects in apoptosis (other than the cardiac defect), and cell biological studies confirm that Bin1 is dispensable for apoptosis in normal cells. However, following oncogenic transformation, a broad requirement for Bin1 in apoptosis is revealed. Notably, oncogene-transformed cells are defective in the apoptotic response to tumor necrosis factor (TNF), DNA damaging agents, and farnesyltransferase inhibitors. All these agents target with some selectivity the neoplastic phenotype, in preclinical models and clinical tests. These findings argue that Bin1 participates in a cell death mechanism(s) that is specific to neoplastic pathophysiology. Preliminary results from mouse crosses support this notion. On wild-type backgrounds, loss of a single Bin1 allele has no discernable effect (up to 10 mos. of age), where on an MMTV-myc "oncomice" background, loss of a single BIn1 allele leads to defective mammary gland function, increased incidence of mammary hyperplasia, and accelerated mammary carcinoma.

In summary, this project has led to the discovery a novel cell death mechanism that may be unique to cancer cells. Loss of this mechanism in breast cancer may provide an important step in malignant progression. Our future efforts focus on activating downstream effectors in the Bin1 pathway, with the aim of restoring this death mechanism and achieving a cancer-selective cell kill in the clinic.

Introduction

An important goal in breast cancer research is to identify better prognostic tools to predict the course and relapse of malignant carcinoma, and to uncover and develop foundations for the development of novel modalities to treat advanced, intractable disease. Malignant breast carcinomas frequently contain deregulated Myc (Shiu *et al.* 1993). Notably, deregulation is most frequently seen in advanced tumors (due to genetic or epigenetic causes) and, where it occurs, signals poor prognosis (Berns *et al.* 1992; Borg *et al.* 1992; Hehir *et al.* 1993; Watson *et al.* 1993). Supporting the importance of the Myc system in breast cancer, the Myc-regulated genes plasminogen activator inhibitor-1 (PAI-1) (Prendergast and Cole 1989; Prendergast *et al.* 1990) and ornithine decarboxylase (ODC) (Bello-Fernandez *et al.* 1993) are also indicators of poor prognosis (Manni *et al.* 1995; Reilly *et al.* 1992; Sumiyoshi *et al.* 1992). The ability of deregulated Myc to drive apoptosis may provide an Achilles' heel in such cells. Indeed, the ability of the anti-breast cancer drug tamoxifen appears to use Myc-mediated death mechanisms to exert its activity (Kang *et al.* 1996). Therefore, unraveling Myc death mechanisms represent one direction to address a major clinical need.

We initiated work in the area of apoptosis by cloning proteins that could interact with the effector domain of Myc. In this manner, we identified the Myc-binding protein Bin1 (formerly termed '99'). The project proposal included preliminary evidence pointing to a role for Bin1 as a breast tumor suppressor gene. In the proposed project, we aimed to:

1. Identify gene mutations and loss of expression in tumor cell lines and primary tumors.
   All tasks completed and findings published.
2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.
   All tasks completed and published. This aim was expanded to generate and analyze mice lacking the Bin1 gene.
3. Mutate Bin1 and assay the mutants for growth inhibitory and/or apoptosis activity.
   All tasks completed and published or submitted for publication. This aim was expanded to clone Bin1-related genes and characterize the function of Bin1 orthologs in the fission yeast S. pombe.

Body

Aim 1. Identify mutations and loss of expression in tumor cell lines and primary tumors

All tasks completed and results published (Sakamuro *et al.*, 1996; Ge *et al.*, 2000). As part of this work we defined the complete structure of the human Bin1 gene and generated a set of Bin1 monoclonal antibodies (Wechsler-Reya *et al*, 1997a; Wechsler-Reya *et al.*, 1997b). We found that Bin1 expression is frequently lost in breast tumor cell lines and primary tumors. The mechanism was not gene deletion but was implied to be gene silencing, based on the loss of both Bin1 protein and message. The Bin1 promoter contains a CpG island (Wechsler-Reya *et al.* 1997b), supporting the hypothesis that the loss of message is due to promoter downregulation through DNA methylation (e.g. similar to p16INK4 in lung cancer). To test this hypothesis we accumulated ~30 matched pairs of normal and primary breast tumor

DNA for testing by methylation-specific PCR, but for technical and manpower reasons this line of investigation (an aim that exceeded original plans) was not completed.

Aim 2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.

All tasks completed and results published (Sakamuro *et al.*, 1996; Elliott *et al.*, 1999; Elliott *et al.*, 2000; Ge *et al.*, 2000). We ultimately completed the work in breast cancer cells using a recombinant adenovirus vector to express Bin1 (Elliott *et al.*, 2000; Ge *et al.*, 2000). In all malignant breast cell lines tested (6/6), where endogenous Bin1 was missing, ectopic expression of Bin1 by the adenoviral vector led to the engagement of a cell death process resembling apoptosis. Notably, in nonmalignant HBL100 cells, where endogenous Bin1 protein was detected, ectopic Bin1 did not cause cell death (Ge *et al.*, 2000). Death was accompanied by cell detachment and a TUNEL-positive apoptotic demise. Cytotoxicity was correlated with Bin1, and not with nonspecific effects of the adenovirus or infection, because similar effects were not produced by infection with uninduced vector or with the Ad-cre virus plus empty vector virus (Elliott *et al.*, 2000; Ge *et al.*, 2000).

We expanded the scope of this Aim to include generation and analysis of Bin1 'knockout' mice. The goal is to determine whether loss of Bin1 in breast tissue promotes breast tumor progression, either in the absence or presence of activated Myc. In previous work, we cloned and characterized the complete mouse Bin1 gene (Mao *et al.* 1998). A gene targeting construct was designed to delete key effector exons that had been identified by structure-function analysis (Elliott *et al.*, 1999). Through a commercial service this blastocysts were injected with this construct and chimeric mice successfully obtained.

Homozygous animals die at approximately E18 of gestation. Some animals are born but are invariably found inviable within several hours. Histological sections reveal a largely normal embryo at all stages of development. However, at later stages of development, cardiac muscle hypertrophy is apparent. The phenotype seems to be pronounced in the ventricles (A. Muller, DuPont Pharmaceuticals Company, pers. comm.). Mice are unusually tolerant of heart hypertrophy during embryonic development (L. Benjamin, Beth Israel Hospital, Harvard University, pers. comm.). This tolerance may explain how animals lacking Bin1 can continue so far past E10 of development when heart function is important. The phenotype observed presumably reflects the inability of cardiac cells to terminally differentiate. This phenotype is consistent with the results of *in vitro* investigations performed in committed C2C12 mouse myoblasts in our laboratory. In this model system, inhibition of Bin1 expression by an antisense strategy led to inhibition of cell cycle withdrawal, terminal differentiation, and apoptosis (Wechsler-Reya *et al.*, 1998; K. Elliott, R. Wechsler-Reya, and G.C.Prendergast, The Wistar Institute, unpublished observations). The defect in cell cycle withdrawal was correlated in the *in vitro* model with a defect in induction of the cell cycle kinase inhibitor p21WAF1 (Mao *et al.*, 1998). This defect could conceivably lead to continuing cell division, consistent with a hypertrophic phenotype.

We have continued the mouse work by monitoring heterozygous animals for tumor formation on wild-type or breast "oncomouse" genetic backgrounds, including MMTV-c-myc and MMTV-neu mice.

Female mice of each strain typically succumb to breast tumors on a multiparous schedule at 5-7 months of age. Preliminary results indicate no evidence of malignancy of other pathologies in heterozygous mice on a wild-type background up to 10 months old. In contrast, heterozygosity on the c-Myc "oncomouse" appears to produce several effects. At first pregnancy, mammary dysfunction manifested by an inability to produce sufficient quantaties of milk is apparent. Many mothers do not nurse. Those that do can not provide sufficient milk and all pups are runted such that most die. Mothers that nurse are prone subsequently to a massive hyperplasia which encompasses all mammary glands. MMTV-c-myc control mice do not exhibit the mammary dysfunction (pups are not runted) but do exhibit some evidence of hyperplasia. We are currently working to determining whether differences are significant. Heterozygous mice appear to come down with carcinomas within 1-3 pregnancies, which is somewhat more rapid than the predicted period for MMTV-c-myc control mice. Carcinoma in situ has been documented in the hyperplastic breast tissue, consistent with the notion of accelerated rates of tumorigenesis and tumor progression. While statistical significance is as yet lacking, if extended and confirmed these findings would provide genetic proof of a tumor suppressor function for Bin1, and would suggest that Bin1 is haploinsufficient for breast cancer suppression.

Aim 3. Mutate Bin1 and assay the mutants for growth inhibitory and/or apoptotic activity.

All tasks completed with results published or submitted (Elliott *et al.*, 1999; Elliott *et al*, 2000; J. DuHadaway, D. Sakamuro, D. Ewert, and G.C. Prendergast, The Wistar Institute and DuPont Pharmaceuticals Company, unpublished results; E. Routhier, DuPont Pharmaceuticals Company, unpublished observations). The c-Myc-binding domain was overexpressed as a dominant inhibitory strategy to probe the requirement for Bin1-c-Myc interaction in proliferation, transformation, and apoptosis. These experiments supported a role in apoptosis by c-Myc but not in proliferation or transformation, consistent with tumor suppressor and cell death roles. In the N-terminal region of Bin1, within its so-called BAR domain, we defined a 5 aa segment the deletion of which abolished antitransforming activity and exhibited dominant inhibitory activity against wild-type Bin1. This mutant is predicted to disrupt a c-Myc-independent effector interaction, and is being used as to counterscreen candidate effector proteins being isolated by genetic and biochemical approaches. This mutant confirmed a requirement for Bin1 in apoptosis but not proliferation or transformation by c-Myc in primary cells. To gain insight into the function of the BAR domain, we have expressed it in bacteria and initiated a collaboration to crystallize it and determine its 3-dimensional structure (E. Laue, Cambridge University).

To gain further insight into Bin1 function, this year we expanded this aim to clone and characterize homologs of Bin1 in the yeast *S. pombe*. We embarked on this project for several reasons. First, work in other laboratories suggested that Bin1 may have roles in actin regulation and endocytosis (Bin1 is related in its BAR domain to amphiphysin, which is implicated in synaptic vesicle endocytosis). How these functions related to nuclear functions of interest to us was unclear. Second, there was evidence in other laboratories that Bin1 orthologs in the yeast *S. cerevisiae* could interact with a BAR domain protein the mammalian ortholog of which was unknown. Third, we were interested in examining functions for Bin1 homologs in *S. pombe* because the cell division mechanisms of that yeast are quite

different from *S. cerevisiae*, with the former resembling mammalian cells more closely. The notion was that Bin1 might display different functions depending on the yeast system.

Consistent with this thinking, we found that the Bin1 homolog in *S. pombe* functioned in cell cycle control but neither actin regulation nor endocytosis. Deletion of this gene product, termed Hob1 (Homolog Of Bin1), could be complemented by human Bin1 but not by its *S. cerevisiae* ortholog (termed Rvs167). This observation established that Bin1 had functions unique to those characterized in other laboratories, consistent with a role in nuclear processes implied by our work. As part of this line of investigation, we cloned the mammalian and *S. pombe* homologs of the *S. cerevisiae* Rvs161 protein, which is the other BAR domain-containing protein in that yeast. These genes were termed Bin3 and Hob3 (Homolog of Bin3), respectively. In contrast to Bin1/Hob1, which differed in function from Rvs167, Bin3/Hob3 were similar in function to Rvs161. Thus, the function of Bin1 has diverged in evolution, a feature possibly reflected by the complex patterns of alternate splicing of Bin1 displayed in mammals. In support of the yeast findings, recent results from the Bin1 "knockout" cells reveals no defect in either actin organization or endocytosis. Thus, we believe that the nuclear functions of Bin1 we have focused on represent a *bona fide* physiological function that is not displayed by amphiphysin. We are using the *S. pombe* system to further mutagenic analyses and exploring the effects of c-Myc in this model, in the hopes of gaining novel insights into protein function, effectors, and signaling processes. Current interest centers on a potential effector role for Bin3 in Bin1 signaling, following preliminary results that Bin1 and Bin3 can interact in principle, similar to Rvs167 and Rvs161, and that Bin3 is localized to the mitochondria where death decisions are made.

Conclusions

The introduction of mouse "knockout" and yeast genetic systems this year offers a near-term opportunity to prove the hypothesis that Bin1 has tumor suppressor and programmed cell death functions. A potential link to mitochondria as well as to nuclear processes are novel features suggested by the latest work in this project. Bin1 investigations seem likely to provide unique insights into cancer cell death pathways, possibly impacting key questions such as why malignant breast cancer cells don't die when they should. We have accelerated efforts to "mine" the Bin1 pathway for therapeutic targets by increasing resources provided for this purpose at DuPont Pharmaceuticals Company. The support of the US Army Breast Cancer Program has been instrumental in supporting what we hope will be not only a highly innovative and important new direction in breast cancer research, but also one that can convert to a better therapeutic approach.

References

Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993). The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc. Natl. Acad. Sci. USA *90*, 7804-8.

Berns, E.M., Klijn, J.G., van, P.W., van, S.I., Portengen, H. and Foekens, J.A. (1992). c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. Cancer Res *52*, 1107-13.

Borg, A., Baldetorp, B., Ferno, M., Olsson, H. and Sigurdsson, H. (1992). c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. Int J Cancer *51*, 687-91.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene **18**, 3564-3573.

Elliott, K., Ge, K., Du, W., and Prendergast, G.C. (2000). The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program. Oncogene **19**, 4669-4684.

Ge, K., DuHadaway, J., Sakamuro, D., Wechsler-Reya, R., Reynolds, C., and Prendergast, G.C. (2000). Losses of the tumor suppressor Bin1 in breast cancer are frequent and reflect deficits in a programmed cell death capacity. Int. J. Cancer *85,* 376-383.

Hehir, D.J., McGreal, G., Kirwan, W.O., Kealy, W. and Brady, M.P. (1993). c-myc oncogene expression: a marker for females at risk of breast carcinoma. J Surg Oncol *54*, 207-9.

Kang, Y., Cortina, R. and Perry, R.R. (1996). Role of c-myc in tamoxifen-induced apoptosis in estrogen-independent breast cancer cells. J. Nat. Canc. Inst. *88*, 279-284.

Manni, A., Wechter, R., Wei, L., Heitjan, D. and Demers, L. (1995). Phenotypic features of breast cancer cells overexpressing ornithine-decarboxylase. J. Cell. Physiol. *163*, 129-36.

Mao, N.C., Steingrimsson, E., J., D., Ruiz, J., Wasserman, W., Copeland, N.G., Jenkins, N.A. and Prendergast, G.C. (1998). The murine Bin1 gene, which functions early in myogenic differentiation, defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. Genomics *submitted*,

Prendergast, G.C. and Cole, M.D. (1989). Posttranscriptional regulation of cellular gene expression by the c-myc oncogene. Mol. Cell. Biol. *9*, 124-134.

Prendergast, G.C., Diamond, L.E., Dahl, D. and Cole, M.D. (1990). The c-myc-regulated gene mrl encodes plasminogen activator inhibitor 1. Mol Cell Biol *10*, 1265-9.

Prendergast, G.C. and Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science *250*, 186-189.

Reilly, D., Christensen, L., Duch, M., Nolan, N., Duffy, M.J. and Andreasen, P.A. (1992). Type-1 plasminogen activator inhibitor in human breast carcinomas. Int J Cancer *50*, 208-14.

Shiu, R.P., Watson, P.H. and Dubik, D. (1993). c-myc oncogene expression in estrogen-dependent and -independent breast cancer. Clin. Chem. *39*, 353-5.

Sumiyoshi, K., Serizawa, K., Urano, T., Takada, Y., Takada, A. and Baba, S. (1992). Plasminogen activator system in human breast cancer. Int J Cancer *50*, 345-8.

Watson, P.H., Safneck, J.R., Le, K., Dubik, D. and Shiu, R.P. (1993). Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. J Natl Cancer Inst *85*, 902-7.

Wechsler-Reya, R., Elliott, K., Herlyn, M., and Prendergast, G.C. (1997a). The putative tumor suppressor Bin1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. Cancer Res. *57,* 3258-3263.

Wechsler-Reya, R., Sakamuro, D., Zhang, J., Duhadaway, J. and Prendergast, G.C. (1997b). Structural analysis of the human BIN1 gene: evidence for tissue-specific transcriptional regulation and alternate RNA splicing. J. Biol. Chem. *272*, 31453-31458.

Bibliography

Publications related to this grant (see Appendices for preprints and reprints)

Mao, N.-C., Steingrimsson, E., Duhadaway, J., Wasserman, W., Ruiz, J., Copeland, N.G., Jenkins, N.A., and Prendergast, G.C. (1999). The murine Bin1 gene functions early in myogenic differentiation and defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. Genomics **56**, 51-58. (Cover article)

DuHadaway, J., Rowe, F., Elliott, K., Mao, N.-C., and Prendergast, G.C. (1999). Bau, a splice form of neurobin-I that interacts with Bin1, inhibits malignant cell growth. Cell Adhes. Comm. **7**, 99-110.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene **18**, 3564-3573.

Ge, K., Duhadaway, J., Sakamuro, D., Wechsler-Reya, R., Reynolds, C., and Prendergast, G.C. (2000). Losses of the tumor suppressor Bin1 in breast cancer are frequent and reflect deficits in a programmed cell death capacity. Int. J. Cancer **85,** 376-383.

Elliott, K., Ge, K., Du, W., and Prendergast, G.C. (2000). The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program. Oncogene **19**, 4669-4684.

Manuscripts related to this grant

DuHadaway, J., Ewert. D., Crouch, D.H., and Prendergast, G.C. Evidence that Bin1 is required for apoptosis by c-Myc. Under revision.

Routhier, E., Abbaszade, I., Burn, T.C., Summers, M., Albright, C., and Prendergast, G.C. Cell cycle but not endocytosis functions for the homologs of Bin1 and its related partner protein Bin3 in fission yeast. In preparation.

Muller, A.J., DuHadaway, J., Mostochuk, J., Du, W., Donover, P.S., and Prendergast, G.C. Bin1 is required for caspase activation and apoptosis in neoplastically transformed cells. In preparation.

Muller, A.J., Mostochuk, J., Farmer, G.E., and Prendergast, G.C. Deletion of the tumor suppressor Bin1 causes cardiac hypertrophy and accelerates Myc-mediated tumorigenesis. In preparation.

Du, W., DuHadaway, J., and Prendergast, G.C. Crucial role for the tumor suppressor Bin1 in apoptosis by farnesyltransferase inhibitors. In preparation.

Related publications

Ge, K., DuHadaway, J., Du, W., Herlyn, M., Rodeck, U., and Prendergast, G.C. (1999). Novel mechanism for elimination of a tumor suppressor: aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. Proc. Natl. Acad. Sci. USA **96**, 9689-9694.

Ge, K., Minhas, F., DuHadaway, J., Mao, N.-C., Wilson, D., Buccafusca, R., Sakamuro, D., Nelson, P., Malkowitz, S.B., Tomaszewski, J.E., and Prendergast, G.C. (2000). Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma. Int. J. Cancer **86**, 155-161.

Ge, K. and Prendergast, G.C. (2000). Bin2, a functionally nonredundant member of the BAR adaptor gene family. Genomics **15,** 210-220.

Related reviews

Sakamuro, D. and Prendergast, G.C. (1998). BIN1 is a Bridging INtegrator-1 of apoptosis mediated by MYC and p53 (in Japanese). In the *Experimental Medicine* series, volume entitled "The New Frontier of p53 Research" (Taya, Y., ed.). Tokyo: Yodosha Co., Ltd.

Sakamuro, D. and Prendergast, G.C. (1999). New Myc interacting proteins: a second Myc network emerges. Oncogene **18**, 2942-2953.

Prendergast, G.C. (1999). Mechanisms of apoptosis by c-Myc. Oncogene **18**, 2966-2986.

Prendergast, G.C. (1999). Myc and Myb: are the veils lifting? Oncogene **18**, 2914-2915.

Related Patents and Patent Applications (14) (all held by The Wistar Institute)

World Intellectual Property Organization (WO) Patent No. WO9634627A1. "Murine and Human Box-Dependent Myc-Interacting Protein (BIN1) and Uses Therefor." Claims: Human and murine Bin1 DNA sequences and vectors. Issued 11/7/96.

Canada Patent No. CA02219775AA. "Murine and Human Box-Dependent Myc-Interacting Protein (BIN1) and Uses Therefor." Claims as above. Issued 11/7/96.

Australia Patent No. AU05674496A1. "Murine and Human Box-Dependent Myc-Interacting Protein (BIN1) and Uses Therefor." Claims as above. Issued 11/21/96.

U.S. Patent No. 5,605,830. "Murine and Human c-Myc Interacting Protein". Claims as above. Issued 2/25/97.

U.S. Patent No. 5,723,581. "Murine and Human Box-dependent Myc Interacting Protein (BIN1) and and Uses Therefor". Claims: Human and murine Bin1 amino acid sequences. Issued 3/3/98.

WO Patent No. WO09808866A1. "BAU, a BIN1-Interacting Protein, and Uses Therefor". Claims: BAU sequences. Issued 3/5/98.

European Patent No. EP00828512A1. "Murine and Human Box-Dependent Myc-Interacting Protein (BIN1) and Uses Therefor." Claims as above. Issued 3/18/98.

Australia Patent No. AU04171797A1. "BAU, a BIN1-Interacting Protein, and Uses Therefor". Claims as above. Issued 3/19/98.

WO Patent No. WO09855151A1. "Box-Dependent Myc-Interacting Protein (BIN1) Compositions and Uses Therefor." Issued 12/10/98.

U.S. Patent No. 5,958,753. "Nucleic Acid Sequences Encoding Bau, a Bin1 Interacting Protein, and Vectors and Host Cells Containing Same". Issued 9/28/99.

U.S. Patent No. 6,048,702. "Murine and Human Box-dependent Myc Interacting Protein (BIN1) and Uses Therefor". Claims: BIN1 antibodies. Issued 4/11/2000.

U.S. Patent Application 09/344,889. "BAU, a BIN1 interacting protein, and Uses Therefor". Claims: BAU amino acid sequences. Allowed.

U.S. Patent Application 09/272,092. "Murine and Human Box-dependent Myc Interacting Protein (BIN1) and Uses Therefor". Claims: Human Bin1 genomic sequences. Pending.

U.S. Patent Application. "Bridging Integrator-2 (Bin2) nucleic acid molecules and proteins and uses therefor". Claims: Bin2 nucleic acid and amino acid sequences. Pending.

Presentations.

Abstracts and lectures on Bin1 and its role in breast cancer and other cancers were presented at 13 conferences and invited seminars since the last report.

2000
- Fox Chase Cancer Center, Annual Symposium (Cancer 2000), Philadelphia PA
- Brown University, Department of Molecular and Cell Biology and Biochemistry, Providence RI
- Princeton University, Department of Molecular Biology, Princeton NJ
- Speaker (*Apoptosis: Mechanisms*), 91st Annual Meeting of the American Association for Cancer Research, San Francisco CA
- University of Pennsylvania Cancer Center (Cancer Genetics Series), Philadelphia PA
- Speaker, Annual Meeting of Environmental Mutagens Society, New Orleans LA

1999
- Speaker (*Apoptosis*), 90th Annual Meeting of the American Association for Cancer Research, Philadelphia PA
- Pew Scholars Meeting, Curacao
- 15th Annual Meeting on Oncogenes, Hood College, Frederick MD
- Fels Institute for Cancer Research and Molecular Biology, Temple University, Philadelphia PA
- Cold Spring Harbor Meeting on Programmed Cell Death, Cold Spring Harbor NY
- Thomas Jefferson University, Department of Dermatology, Philadelphia PA

• Delaware Valley Prostate Cancer Symposia, Philadelphia PA

Personnel supported on project (throughout entire term of grant, including partial support)

Graduate Trainee	*Term*	*Current Career Status*
Katherine Elliott, Ph.D.	1994-1999	Family leave

Postdoctoral Trainees	*Term*	*Current Career Status*
Daitoku Sakamuro, Ph.D.	1994-1999	Assistant Professor, Purdue University
Robert Wechsler-Reya, Ph.D.	1995-1997	Assistant Professor, Duke University
Nien-Chen Mao, Ph.D.	1997-1998	Clinical Staff Scientist, DuPont Pharmaceuticals
Kai Ge, Ph.D.	1997-2000	Postdoctoral Fellow, Rockefeller University

Int. J. Cancer: **85,** 376–383 (2000)

Publication of the International Union Against Cancer

LOSSES OF THE TUMOR SUPPRESSOR BIN1 IN BREAST CARCINOMA ARE FREQUENT AND REFLECT DEFICITS IN PROGRAMMED CELL DEATH CAPACITY

Kai GE[1], James DUHADAWAY[2], Daitoku SAKAMURO[1], Robert WECHSLER-REYA[1], Carol REYNOLDS[3] and George C. PRENDERGAST[1,2]*

[1]*The Wistar Institute, Philadelphia, PA, USA*
[2]*Dupont Pharmaceuticals, Glenolden Laboratory, Glenolden, PA, USA*
[3]*Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA, USA*

Oncogenic activation of MYC occurs often in breast carcinoma and is associated with poor prognosis. Loss or inactivation of mechanisms that restrain MYC may therefore be involved in tumor progression. In this study, we show that the MYC-interacting adaptor protein BIN1 is frequently missing in malignant breast cells and that this loss is functionally significant. BIN1 was expressed in normal and benign cells and tissues but was undetectable in 6/6 estrogen receptor–positive or estrogen receptor–negative carcinoma cell lines examined. Similarly, complete or partial losses of BIN1 were documented in 30/50 (60%) cases of malignant breast tissue analyzed by immuno-histochemistry or RT-PCR. Abnormalities in the organization of the *BIN1* gene were apparent in only a minority of these cases, suggesting that most losses were due to epigenetic causes. Nevertheless, they were functionally significant because ectopic BIN1 induced programmed cell death in malignant cells lacking endogenous BIN1 but had no effect on the viability of benign cells. We propose that loss of BIN1 may contribute to breast cancer progression by eliminating a mechanism that restrains the ability of activated MYC to drive cell division inappropriately. *Int. J. Cancer* 85:376–383, 2000.
© *2000 Wiley-Liss, Inc.*

The genetic events underlying the development of sporadic breast cancer are not fully defined. Inactivation of the *BRCA1* and *BRCA2* genes contributes to a significant fraction of familial breast cancers, but these genes are altered only rarely in sporadic forms of the disease. Identification of other genes which have sustained loss of function may help to provide insight into mechanisms of progression as well as promote novel opportunities for molecular approaches to diagnosis, prognosis or therapy. One approach to this problem is to examine roles for the regulatory and effector elements of systems that have fundamental significance in cancer, such as the growth-regulatory network controlled by MYC (Sakamuro and Prendergast, 1999). MYC has a crucial role in mitogenesis of many types of normal and neoplastic cells, and it is oncogenically activated in a wide variety of human malignancies including breast cancer (Nass and Dickson, 1997). In sporadic breast cancer, MYC over-expression occurs in as many as 50% of cases and has been widely associated with poor prognosis (Berns *et al.,* 1992; Borg *et al.,* 1992; Watson *et al.,* 1993). However, despite its potential importance, the mechanisms which promote MYC activation during breast tumor progression are not fully understood (Nass and Dickson, 1997). MYC lies at the crossroads of 2 signaling networks which target its C-terminal DNA-binding domain and its N-terminal transcriptional transactivation domain (Sakamuro and Prendergast, 1999). The major player in the C-terminal network is MAX, the crucial b/HLH/Z partner protein which heterodimerizes with MYC and mediates its ability to specifically recognize DNA. However, MAX alterations do not occur in human cancer. A number of novel N-terminal-interacting proteins which constitute a second Myc network have been identified (Sakamuro and Prendergast, 1999). One of these, BIN1, is a ubiquitously expressed adaptor protein with features of a tumor suppressor (Sakamuro *et al.,* 1996; Elliott *et al.,* 1999). Previous work indicated that BIN1 was missing in MCF7 cells and in a small panel of primary tumors examined (Sakamuro *et al.,* 1996), but the frequency and biological relevance of these events was uncertain. In this study, we present evidence that BIN1 expression is frequently lost in sporadic breast cancer and that such losses are biologically significant. Our findings suggest that loss of BIN1 contributes to the progression of breast cancer by allowing cells to activate MYC without apoptotic penalty.

MATERIAL AND METHODS

Tissue culture

Human cell lines were obtained from the ATCC (Rockville, MD), with the exception of SK-BR-5, which was obtained from Dr. D. Herlyn (The Wistar Institute). Non-malignant HBL-100 breast epithelial cells; the breast carcinoma cell lines BT20, MCF7, MDA-MB-468 and SK-BR-3; WI-38 and IMR90 diploid fibroblasts; and 293 embyonic kidney epithelial cells were maintained in DMEM supplemented with 10% FBS (Life Technologies, Gaithersburg, MD) and 50 U/ml penicillin and streptomycin (Fisher, Pittsburgh, PA). The breast carcinoma cell lines T47D and ZR-75–1 were maintained in RPMI 1640 that was supplemented similarly. Viable cell counts were obtained by Trypan blue exclusion.

Recombinant adenoviruses

The adenoviral cre-loxP-inducible gene expression system used in this study has been described (Anton and Graham, 1995). Briefly, the luciferase cDNA in the vector pMA19 (Anton and Graham, 1995) was replaced with the *BIN1* cDNA 99fE (Sakamuro *et al.,* 1996). The resulting plasmid was co-transfected with ClaI-digested dl7001 adenovirus DNA into 293 cells to obtain the recombinant adenovirus Ad-MABIN1 by homologous recombination (Davis and Wilson, 1996). In this virus, the *BIN1* cDNA is conditionally expressed under the control of the cytomegalovirus (CMV) promoter, from which it is separated by a loxP-flanked stuffer sequence. BIN1 expression is suppressed in the unrearranged virus because the stuffer region includes stop codons in all 3

Grant sponsor: Wistar Institute; Grant number: CA10851; Grant sponsor: US Army Breast Cancer Research Program; Grant number: DAMD17–96–1-6324; Grant sponsor: American Cancer Society; Grant number: CN-160.

Kai Ge and James Duhadaway contributed equally to this study.

Daitoku Sakamuro's current address is: Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Purdue University, West Lafayette, IN 47907-1333, USA.

Robert Wechsler-Reya's current address is: Department of Developmental Biology, Stanford University, Stanford, CA 94305, USA.

Carol Reynold's current address is: Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

*Correspondence to: Dupont Pharmaceuticals, Glenolden Laboratory, 500 S. Ridgeway Avenue, Glenolden, PA 19036, USA. Fax: +1–610–237–7847. E-mail: george.c.prendergast@dupontpharma.com

Received 17 June 1999; Revised 16 August 1999

reading frames. Upon co-infection of cells with Ad-MABIN1 and Ad-cre [adenovirus expressing Cre site-specific recombinase from bacteriophage P1 (Anton and Graham, 1995)], the intervening stuffer region was removed by Cre-mediated excision, causing BIN1 expression. Ad-vect is a similar control recombinant adenovirus except that it contains no transgene (Davis and Wilson, 1996). For adenoviral infection, cells were plated at 2 to 4 $\times$ 10^5 per well in 6-well tissue culture plates. Forty hours later, after cell density reached approx. 5 $\times$ 10^5 per well, equivalent amounts of Ad-cre plus Ad-vect, Ad-vect plus Ad-MABIN1 or Ad-cre plus Ad-MABIN1 viruses were added at 50 multiplicities of infection (m.o.i.) each (BT20), 100 m.o.i. each (MCF7, MDA-MB-468, SK-BR-3, T47D and ZR-75–1) or 200 m.o.i. each (HBL-100) in 1 ml of a 1:1 mixture of DMEM and RPMI 1640 supplemented only with 4 μg/ml polybrene (Sigma, St. Louis, MO). The m.o.i. employed were determined empirically for each cell line to achieve >90% infection of the target cell population, through experiments using a recombinant adenovirus expressing β-galactosidase. After incubation for 3 hr, cells were washed once with a 1:1 mixture of unsupplemented DMEM and RPMI 1640 and then fed with normal culture medium. Cells were harvested or photographed using phase contrast microscopy as indicated at times afterward.

Flow cytometry

Floating and adherent cells were harvested 48 hr after infection and fixed in 1% paraformaldehyde/PBS followed by 80% ethanol. Samples were incubated for 1 hr at 37(C in TdT reaction buffer (Roche, Indianapolis, IN), incubated for 30 min at room temperature with fluoresceinated streptavidin (DuPont, Wilmington, DE) and stained 30 min with propidium iodide at room temperature. Flow-cytometric analysis was performed on a EPIC/XL cell analyzer.

RT-PCR

Total RNA was isolated as described (Shiozawa *et al.,* 1990) from frozen breast tissues obtained from the Cooperative Human Tissue Network (Philadelphia, PA). Total cytoplasmic RNA was prepared from cell lines by standard methods. For cell line analyses, primers and reaction conditions used for RT-PCR have been described (Wechsler-Reya *et al.,* 1997*b*). For tissue analyses, quantitative RT-PCR was performed as follows. One microgram total cytoplasmic RNA in 5 μl water was denatured by 5 min incubation at room temperature with 2 μl 0.1 M methylmercury hydroxide. To this RNA was added 2.5 μl 0.7 M β-mercaptoethanol and 0.5 μg random hexanucleotides in 1 μl aqueous solution. Following a 2 min incubation at 70°C, RNAs were incubated on wet ice and 2.0 μl 5 mM dNTPs, 0.5 μl RNase inhibitor (Promega, Madison, WI), 4 μl 5× RT buffer, 2 μ l 0.1 M DTT and 1.0 μl Mo-MLV reverse transcriptase (Life Technologies) were added. Reactions were incubated for 60 min at 37°C and then stopped by a 5 min incubation at 95°C. This RT product was used as template to amplify BIN1 or β-actin cDNA by PCR. For BIN1 (exons 1–5), the 5′ primer was 5′-AAAGATCGCCAGCAACGTGC and the 3′ primer was 5′-CTGGTGGTAATCCATCCACAGC. For β-actin (exons 3–4), the 5′ primer was 5′-GGTGGGCATGGGTCAGAAGG and the 3′ primer was 5′-GCAGCTCGTAGCTCTTCTCC. Reactions were performed in 100 μl containing 200 ng template, 50 pmol each primer, 0.4 mM each dNTP, 1× Taq PCR buffer and 2.5 U Taq polymerase (Roche). PCR was performed by 2 min incubation at 96°C and then 26 cycles (BIN1) or 22 cycles (β-actin) of 30 sec 96°C/45 sec 61°C/45 sec 72°C followed by a final extension of 10 min at 72°C. Products were purified from 1.4% TBE agarose gels and in some cases subjected to direct DNA sequencing. For the tumor RNA samples indicated by asterisks in Figure 1*c*, in which the entire coding region of BIN1 cDNA was cloned by RT-PCR and subjected to direct DNA sequencing, 3 separate PCRs were performed using primers and conditions that have been described (Wechsler-Reya *et al.,* 1997*b*).

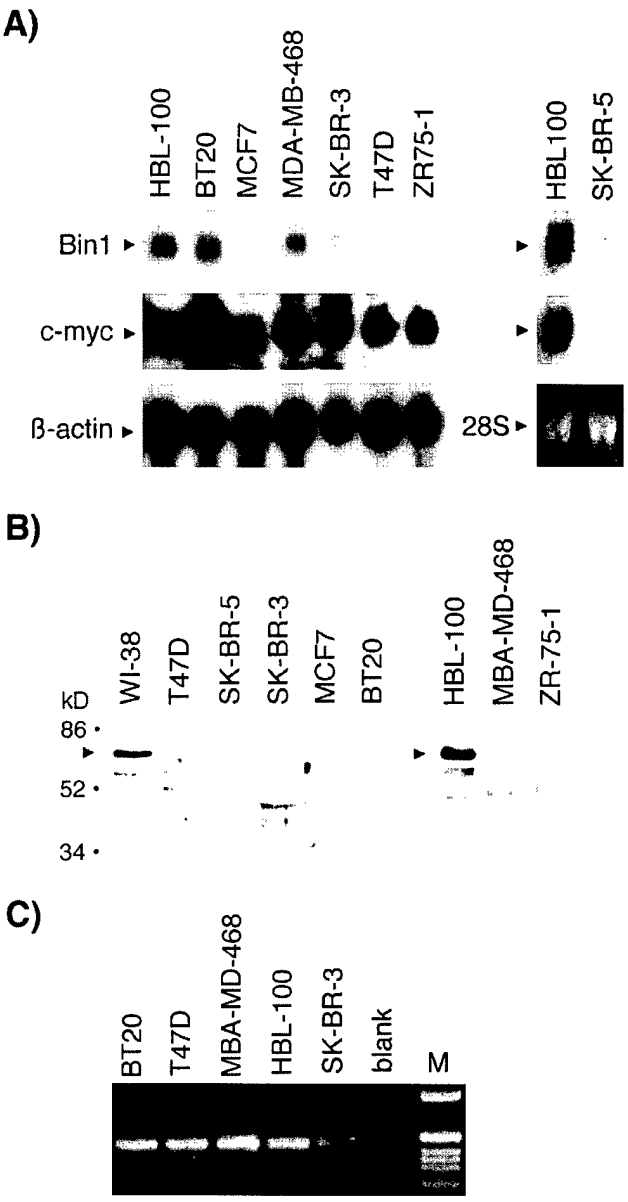


FIGURE 1 – Frequent loss of BIN1 expression in breast carcinoma cell lines. *(a)* Northern analysis. Total cytoplasmic RNA isolated from the cell lines indicated was analyzed using probes for BIN1, MYC and β-actin. *(b)* Western analysis. Cell extracts were prepared from the cell lines indicated and subjected to SDS-PAGE and Western blotting using anti-BIN1 MAb 99D (Wechsler-Reya *et al.,* 1997*a*). *(c)* RT-PCR analysis. Total cytoplasmic RNA isolated from the cell lines indicated was analyzed as described (Wechsler-Reya *et al.,* 1997*b*). Products derived from region II, which includes the central domain encoded by exons 7–11, were fractionated on agarose gels, stained with ethidium bromide and photographed. SK-BR-3 exhibits an aberrant product in addition to the expected wild-type product (arrow). M, DNA size marker.

Southern and Northern analyses

High-m.w. genomic DNA from human breast tissues and cell lines was isolated as described (Herrmann and Frischauf, 1987). For Southern analysis, 5 μg genomic DNA were digested overnight at 37°C with 30 U Hind III restriction endonuclease (Roche), fractionated by agarose electrophoresis, blotted by capillary transfer to Duralon membranes (Stratagene, La Jolla, CA) and hybridized as described (Church and Gilbert, 1984) with ^{32}P-labeled

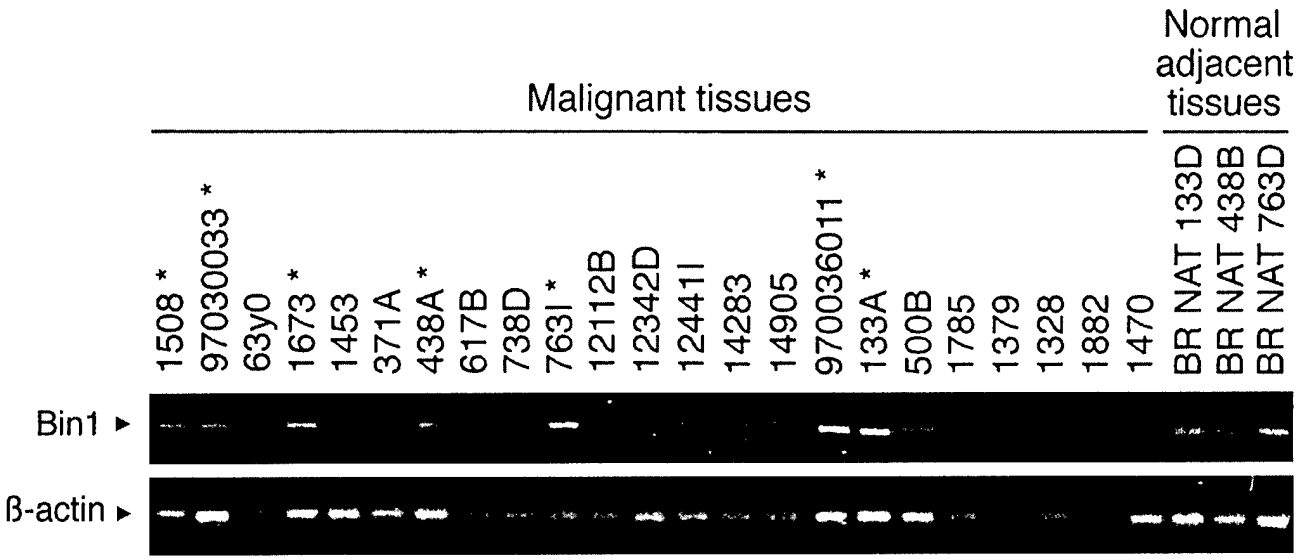


FIGURE 2 – Frequent loss of BIN1 RNA in primary breast tumors. Total RNA isolated from normal and malignant breast tissues was analyzed as described (Wechsler-Reya *et al.*, 1997*b*), using primers which amplify BIN1 or β-actin messages. The complete BIN1-coding regions of samples marked with an asterisk were cloned by RT-PCR, and their DNA sequence was determined as described (Wechsler-Reya *et al.*, 1997*b*).

TABLE I – IMMUNO-HISTOCHEMICAL ANALYSIS OF BIN1 IN BREAST TISSUE

Tissue analyzed or histological diagnosis	n[1]	–	+/–	+	++
Normal ductal epithelium[2]	4	0	0	0	4
Lactating adenoma	1	0	0	0	1
Metaplastic carcinoma	1	0	1	0	0
Malignant phyllodes	1	1	0	0	0
Tubular carcinoma	1	0	0	0	1
Infiltrating lobular carcinoma	1	1	0	0	0
Infiltrating ductal carcinoma	23	9	2	5[3]	7[3]
Total malignant tissues	27	11 (41%)	3 (11%)	5 (18%)	8 (30%)

Frozen benign or malignant breast tissue was sectioned and subjected to immuno-histochemistry as described in Material and Methods. The relative proportion of cell nuclei in the section which stained with BIN1 MAb 99D (Wechsler-Reya *et al.*, 1997*a*) was scored as follows: –, negative; +/–, <10% cells staining; +, 10% to 70% cells staining; ++, >70% cells staining.

[1]n, number of specimens examined.–[2]Tissue derived from reduction mammoplasty.–[3]Included 2 tumors exhibiting both nuclear and cytosolic staining.

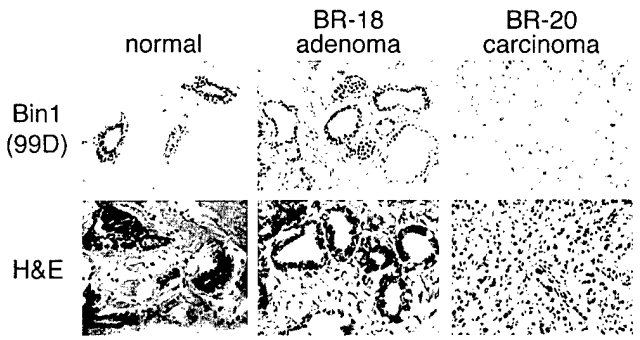


FIGURE 3 – Immuno-histochemical analysis of BIN1 in primary breast tumors. Representative patterns of BIN1 staining in frozen sections of normal, benign adenoma and malignant breast tissues documented in Table I are presented. Lower panels are histological sections stained with standard hematoxylin–eosin (H&E).

probes generated by the random priming method (Roche). Probes were an approx. 1.5 kb Eco RI fragment from the 99fE cDNA including the coding region of BIN1 (Sakamuro *et al.*, 1996) or an approx. 1.6 kb Xba I fragment of the human *BIN1* gene encompassing exon 1 and the promoter region (Wechsler-Reya *et al.*, 1997*b*). Blots were washed at a final stringency of 0.2× SSC at 60°C and subjected to autoradiography at –80°C from 1 to 3 days. For Northern analysis, total cytoplasmic RNA was prepared from tissues and cell lines as described (Prendergast and Cole, 1989; Shiozawa *et al.*, 1990), fractionated on formaldehyde agarose gels, blotted and hybridized with ^{32}P-labeled human MYC (Prendergast *et al.*, 1991) or BIN1 (99fE) cDNA probes, as described above.

Western analysis

Cell lysates were prepared in NP40 buffer and analyzed by SDS-PAGE and Western blotting using standard methods. Blots were probed with BIN1 monoclonal antibody (MAb) 99D (Wechsler-Reya *et al.*, 1997*a*) and developed using a 1:5,000 dilution of horseradish peroxidase–conjugated goat anti-mouse IgG (Roche) and a chemiluminescence kit, employing the conditions suggested by the vendor (Pierce, Rockford, IL).

Immuno-histochemistry

Frozen cases of normal and malignant breast tissues were obtained from the Department of Pathology and Laboratory Medicine at the Hospital of the University of Pennsylvania (Philadelphia, PA) and from the Department of Pathology at the University of Michigan Medical School (Ann Arbor, MI). Frozen tissues were sectioned and processed for immuno-histochemistry using anti-BIN1 MAb 99D (Wechsler-Reya *et al.*, 1997*a*), which efficiently stains frozen, but not formalin-fixed, tissues (Sakamuro *et al.*, 1996). Sections were warmed to room temperature, washed twice with PBS, fixed in 4% paraformaldehyde/PBS for 30 min at 4°C and rinsed once with water and then twice with PBS. Tissues were permeabilized in 0.1% Triton X-100 for 10 min, washed twice with PBS and incubated for 15 min in 1% H_2O_2 in methanol to quench endogenous peroxidase. After washing for 5 min each in PBS and then PBS/0.1% BSA, samples were incubated for 30 min

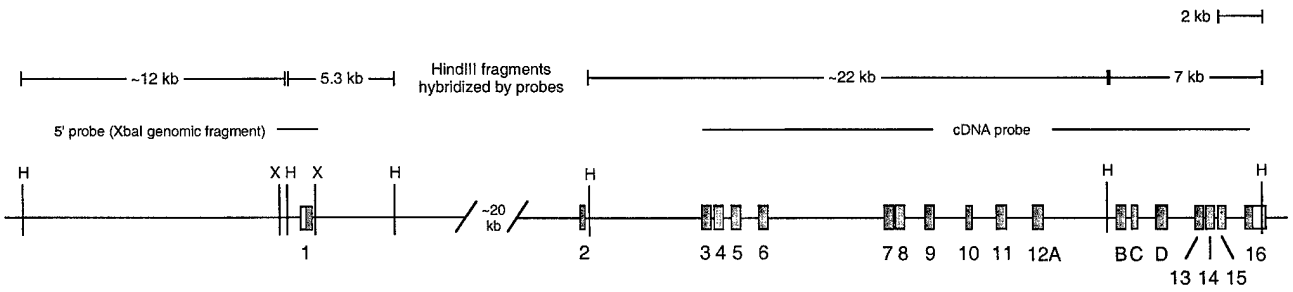


FIGURE 4 – Organization of the human *BIN1* gene. Exon–intron organization of the human *BIN1* gene and its 5′ upstream region was characterized previously by Wechsler-Reya *et al.* (1997*b*). Exon 1 and the promoter region are separated from the main part of the gene by >20 kb. The upper part of the figure depicts the HindIII restriction fragments hybridized by a cDNA probe (for the main body of the gene) or by an XbaI genomic restriction fragment probe (for the exon 1 promoter region). H, HindIII; X, XbaI.

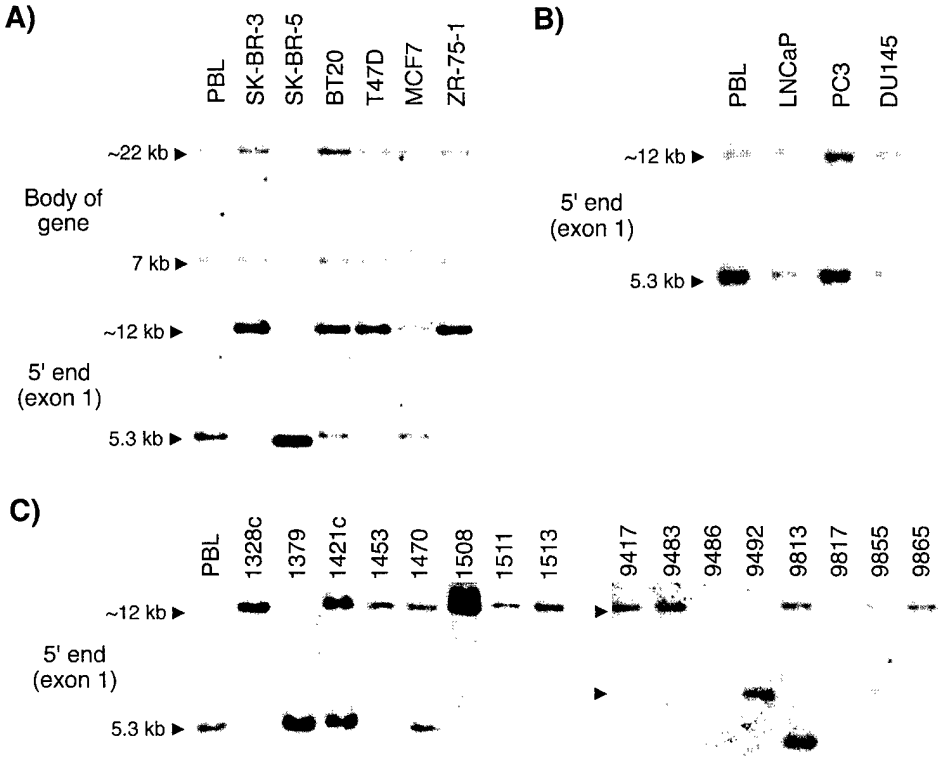


FIGURE 5 – Aberrant organization of the *BIN1* gene in breast carcinomas and primary tumors. Genomic DNA was isolated from normal PBLs or from the cell lines and primary tumors indicated, digested with HindIII endonuclease, fractionated on agarose gels and subjected to Southern blotting and hybridization with ^{32}P-labeled cDNA or genomic probes as noted in Figure 4. *(a)* Carcinoma cell lines. The blot was hybridized with the cDNA probe that recognizes the body of the gene (top panel) and then stripped and rehybridized with the genomic probe that recognizes the 5′ region of the gene (bottom panel). *(b)* Wild-type organization of the 5′ end of the *BIN1* gene in prostate carcinoma cells. Hybridization data using the 5′ genomic probe are shown. *(c)* Primary tumors. Hybridization data using the 5′ genomic probe are shown.

with anti-BIN1, washed twice in PBS and incubated for an additional 30 min with a 1:200 (v/v) dilution of biotin-conjugated AffiniPure goat anti-mouse IgG(H+L) (Jackson ImmunoResearch, West Grove, PA). Slides were developed by 30 min incubation with peroxidase-conjugated streptavidin (Dako, Carpinteria CA) diluted at 1:200 in PBS/0.1% BSA, washed for 5 min in PBS 3 times and then flooded for 5 min with substrate (Peroxidase Substrate DABkit; Vector, Burlingame, CA). After rinsing in water, slides were counterstained with 0.04% light green in acidified water or dilute hematoxylin for 1 min, dehydrated and cover-slipped. Stained slides were examined and scored for overall mean extent of nuclear staining. Extent of staining was evaluated on a scale of 0 to 2+ (0 = 0%, +/– = <10%, + = 10% to 70%, ++ = >70%).

RESULTS

Loss of BIN1 occurs frequently in breast cancer

Previously, we showed that MCF7 cells and 3/6 primary human tumors examined lacked detectable BIN1 message (Sakamuro *et al.,* 1996). To determine the extent of such losses and to assess the mechanism, we examined the status of BIN1 expression in a set of 7 human carcinoma cell lines and 50 cases of normal and malignant breast tissue. Levels in cell lines were determined in non-malignant HBL-100 cells; the estrogen-dependent carcinoma cells lines T47D, ZR-75–1 and MCF7; and the estrogen-independent carcinoma cell lines BT20, MDA-MB-468, SK-BR-3 and SK-BR-5. BIN1 RNA levels in 5/7 of these cell lines were reduced or undetectable relative to HBL-100 (Fig. 1*a*, upper panels). Elevated

MYC levels were observed in all cell lines, with the exception of SK-BR-5, where MYC message was undetectable (Fig. 1*a*, middle panels). The differences in message levels were not due to differences in RNA loading, as shown by ethidium bromide staining of Northern gels or hybridization of Northern blots with β-actin cDNA (Fig. 1*a*, lower panels). Western analysis using the anti-BIN1 MAb 99D (Wechsler-Reya *et al.*, 1997*a*) confirmed and extended the deficits in BIN1 expression in malignant cells (Fig. 1*b*). In particular, BT20 and MDA-MB-468 lacked wild-type protein, though they expressed normal levels of BIN1 message. While the basis for this difference was unclear, these data increased the extent of losses of wild-type BIN1 protein to all malignant cell lines examined. In SK-BR-3 cells, a truncated polypeptide was detected. RT-PCR analysis of the cell lines which expressed detectable message levels (HBL-100, BT20, T47D and SK-BR-3) revealed normal message structure in all cell lines except for SK-BR-3, which also expressed an aberrantly spliced isoform that correlated with production of the truncated polypeptide (Fig. 1*c*). In contrast to the lack of BIN1 expression in malignant cells, BIN1 polypeptides of appropriate size were detected in WI-38 diploid fibroblasts and non-malignant HBL-100 cells.

We next examined the levels of BIN1 message and protein in breast tumors, to confirm the extent of losses in primary malignancies and to rule out the trivial possibility that losses were due to *in vitro* cell line establishment. BIN1 messages were examined in RNAs isolated from a panel of 3 benign and 23 malignant breast tissues by RT-PCR (Fig. 2). All 3 benign tissues examined were positive for expression, but 16/23 (70%) malignant tissues exhibited reduced or undetectable levels of BIN1 message relative to normal tissues. The complete coding regions of the messages in the 7 samples which exhibited robust expression (asterisks in Fig. 2) were cloned by RT-PCR and the DNA sequence determined as described previously (Wechsler-Reya *et al.*, 1997*b*). No alterations relative to the wild-type BIN1 sequence were observed (data not shown), suggesting that alterations of BIN1 in breast cancer related predominantly to loss of expression rather than polypeptide mutation. To confirm losses at the level of protein accumulation, we performed an immuno-histochemical analysis of 5 benign and 27 malignant cases of breast tissue (Table I, Fig. 3). This analysis included 4 normal tissues derived from reduction mammoplasty, 1 case of lactating adenoma, 23 cases of infiltrating ductal adenocarcinoma and 1 each of infiltrating lobular carcinoma, tubular carcinoma, metaplastic carcinoma and malignant phyllodes (a relatively rare tumor with high mitotic rate, infiltrative borders, stromal atypia and over-growth). All benign tissues, including the lactating adenoma, exhibited uniform and robust staining of BIN1 in cell nuclei, consistent with the localization of BIN1 in other normal or benign cell types (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997*a*, 1998). In contrast, complete or partial losses of BIN1 were observed in 11/27 (41%) and 3/27 (11%) malignant cases, respectively. Figure 3 presents examples of the positive staining patterns observed in normal tissue and benign adenoma as well as the most common negative pattern seen in infiltrating ductal carcinoma. In cases of malignant tissue positive for BIN1, 4/12 exhibited staining in the cytosol as well as the nucleus of neoplastic cells (data not shown). In addition, some positive cells exhibited staining of punctate subnuclear domains instead of nucleoplasm, reminiscent of abnormal patterns seen in other malignant cells, such as SAOS-2 osteosarcoma cells (Wechsler-Reya *et al.*, 1997*a*). Further investigation is required to determine the functional impact of these altered staining patterns. To assess the possibility of a correlation between BIN1 status and other pathological markers, we examined the nuclear grade, histological grade, ploidy, lymph node positivity and status of ERBB2 (HER2), progesterone receptor and estrogen receptor in a subset of 15 malignant cases, but no correlation with these markers was apparent (data not shown). Nevertheless, when taken together, the results of the RT-PCR and immuno-histochemical analyses indicated that BIN1 was greatly reduced or undetectable in 60% of the 50 malignant cases examined. We concluded that loss of BIN1 occurred frequently in neoplastic breast disease.

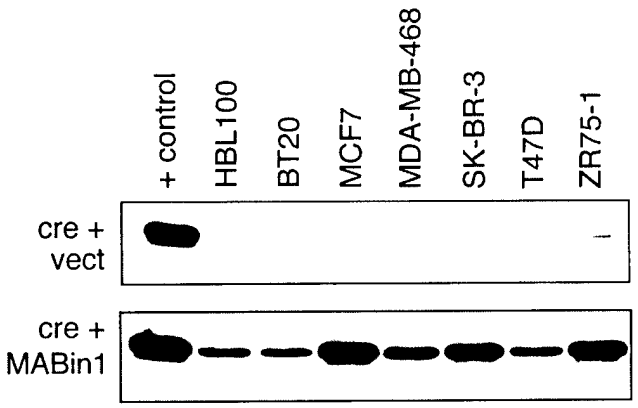


FIGURE 6 – Inducible expression of BIN1 in adenovirus-infected breast cells. Cell extracts were prepared 20 hr after infection with Ad-cre plus Ad-vect (upper panel) or Ad-cre plus Ad-MABIN1 (lower panel), and equivalent amounts were subjected to Western analysis with anti-BIN1 MAb 99D. The positive control for expression is an extract derived from a BIN1-expressing human melanoma cell line.

Status of the BIN1 gene in breast carcinoma cell lines and tumors

Southern analysis was performed to compare the organization of the human *BIN1* gene in malignant and benign cells, to determine whether losses in expression were accompanied by any genetic abnormalities. Genomic DNA was isolated from normal peripheral blood lymphocytes (PBLs), as a control for wild-type organization, as well as from a set of malignant breast cell lines and 24 primary breast tumors. Figure 4 presents the organization of the *BIN1* gene as previously determined (Wechsler-Reya *et al.*, 1997*b*) and the Southern analysis strategy used in this study. Restriction of genomic DNA by HindIII endonuclease generates 2 contiguous fragments of 22 kb and 7 kb that encompass the body of the gene recognized by a *BIN1*-coding region cDNA probe; the same digestion also generates 2 contiguous fragments of 12 kb and 5.3 kb encompassing the 5′ upstream region recognized by an XbaI restriction fragment that includes exon 1 and promoter sequences (Fig. 4). Genomic DNA from normal PBLs produced the expected gel band patterns for both regions of the gene (Fig. 5*a*). In contrast, aberrant band patterns were noted in genomic DNA isolated from SK-BR-5, which apparently sustained a homozygous deletion of the *BIN1* gene consistent with the lack of message or protein production. SK-BR-3 exhibited both normal and aberrant fragments within the body of the gene, consistent with the production of an aberrant splice product and a truncated polypeptide (Fig. 1). Other cell lines exhibited wild-type organization of the body of the gene, as did all primary tumors examined (data not shown). However, additional examples of abnormal gene organization were seen in carcinoma cells and primary tumors at the 5′ end of the gene (Fig. 5*a,b*). SK-BR-5 exhibited a single aberrant band consistent with the likelihood of a large deletion within the *BIN1* gene in this cell line. SK-BR-3, T47D and ZR-75–1 exhibited loss of the 5.3 kb fragment that includes exon 1 and the proximal region of the promoter. While polymorphism was not ruled out, similar abnormalities were not observed in a set of prostate cancer cell lines, the *BIN1* organization of which was identical to that observed in PBLs (Fig. 5*b*). Interestingly, 3/4 of the carcinoma lines that exhibited aberrant 5′ organization also lacked detectable message. Analysis of primary tumors showed that 8/16 tumors had similar aberrant 5′ banding patterns. An additional 3/16 tumors (1508, 9492 and 9813) exhibited bands of unique mobility where polymorphism as a cause could be ruled out. Using this type of benchmark, a conservative interpretation was that at least 1/6 of the cancer cell lines and 3/16 of the primary tumors had mutations in *BIN1*. However, since a

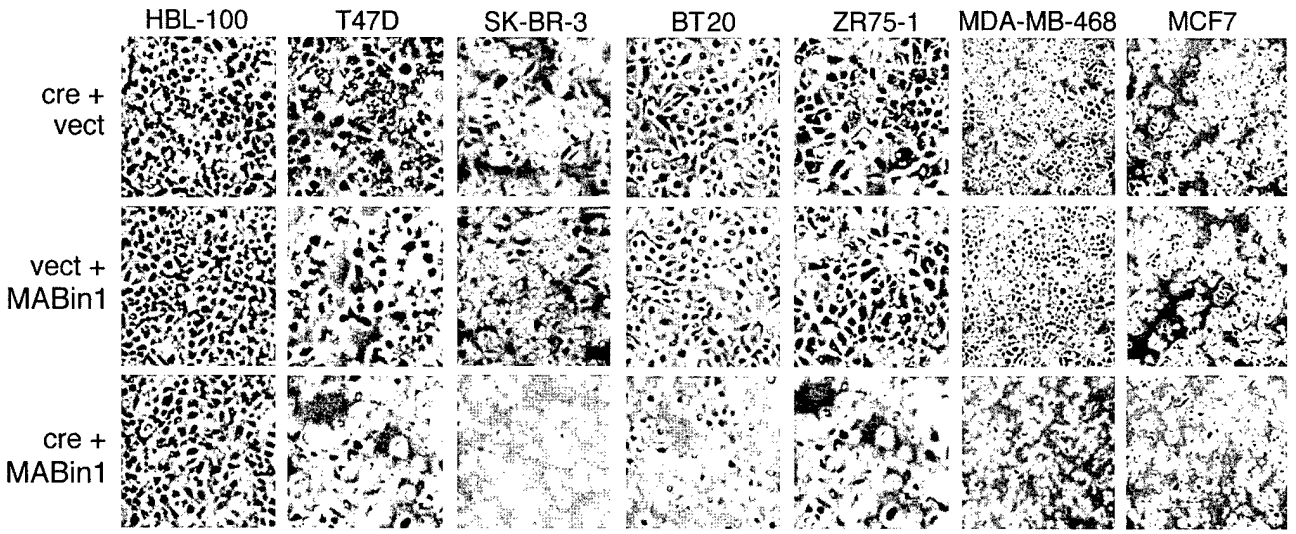


FIGURE 7 – Cytopathic effect of ectopic BIN1. Cells were infected with the viruses indicated in Figure 3 and photographed 48 hr later using phase contrast microscopy.

greater proportion of malignant cells exhibited losses in expression than clear genetic abnormalities, it appeared that loss of BIN1 expression in breast neoplastic disease was due to epigenetic as well as genetic causes.

BIN1 induces programmed cell death in malignant cells independently of estrogen receptor status

To address the functional significance of BIN1 loss, an adenoviral vector system was used to examine the consequences of ectopic re-expression of BIN1 in a panel of the breast tumor cell lines analyzed. The system used an inducible vector that allows conditional expression based on the cre-loxP system, which allows one to rule out toxic effects of adenovirus or uninduced vector DNA. The construction and characterization of this system will be described in detail elsewhere. Briefly, the full-length *BIN1* cDNA was cloned into a standard adenoviral vector downstream of a stuffer fragment flanked by loxP sites. Co-infection with a recombinant adenovirus expressing Cre recombinase (Ad-cre) results in removal of the stuffer fragment and ligation of the *BIN1* cDNA immediately downstream of the CMV early-region enhancer/promoter, causing it to be expressed. The m.o.i. required to infect ≥90% of the target population for each of the breast cell lines was determined using a β-galactosidase vector (Ad-lacZ) and standard methods of staining cells with X-gal to monitor β-galactosidase expression. In this manner, an m.o.i. of 100 was found to be sufficient to infect each cell line used (data not shown). Expression of BIN1 from the adenoviral vector was robust in all cells co-infected with Ad-cre (Fig. 6).

Expression of BIN1 in the malignant cells was correlated with the appearance of a cytopathic phenotype that was characterized by substratum detachment (Fig. 7). Uninduced or control viruses did not produce similar effects at the m.o.i. employed. The most pronounced cytopathic phenotype occured in estrogen receptor–positive ZR-75–1 cells and estrogen receptor–negative SK-BR-3 or MDA-MB-468 cells; however, all of the malignant cell lines tested exhibited this phenotype to various degrees. Non-malignant HBL-100 cells were not affected by ectopic BIN1 expression. Consistent with a specific effect on malignant cells, ectopic BIN1 causes similar effects on human melanoma cells but not fetal melanocytes (Ge *et al.,* 1999) and IMR90 diploid fibroblasts are not affected by ectopic BIN1 (data not shown). To confirm that the cytopathic phenotype observed in malignant cells was correlated with induction of cell death, an explicit determination of viability was made by Trypan blue exclusion in cells harvested 48 hr after adenoviral infection. With the exception of non-malignant HBL-100 cells, all of the cell lines exhibited a reduction in viability in response to BIN1 expression (Fig. 8*a*). A correlation was noted between the extent of cell death and the cytopathic phenotype. Estrogen receptor–positive T47D and MCF7 cells and estrogen receptor–negative BT20 cells were the least affected, but their viability was still reduced significantly at the time point tested. The effect on MCF7 was consistent with the BIN1-induced growth inhibition documented previously (Sakamuro *et al.,* 1996). The lack of correlation between estrogen receptor status and BIN1 response was consistent with a similar lack of correlation between the status of BIN1 and the estrogen receptor indicated by the immunohistochemical analysis. Flow cytometry was performed to determine if cell death was due to apoptosis as defined by TUNEL assay and DNA degradation. Virally infected cells harvested 48 hr later were processed for TUNEL reaction and propidium iodide staining and analyzed by flow cytometry. In this assay, cells undergoing programmed cell death are identified on the basis of the presence of sub-G_1 phase DNA (Schwartz and Osborne, 1995). Table II presents the proportion of cells in the sub-G_1 phase fraction which sustained extensive DNA degradation. The basal level of sub-G_1 phase cells in the HBL-100 population examined was not altered by ectopic expression of BIN1. In contrast, following ectopic expression of BIN1, all malignant cell lines exhibited an increase in the proportion of cells in the sub-G_1 fraction, with a good correlation between the relative extent of DNA degradation and loss of viability. DNA degradation consistent with programmed cell death was confirmed by TUNEL assay in HBL-100 and MDA-MB-468 cells. As expected, MDA-MB-468 cells were labeled by TUNEL reaction and showed an increase in the proportion of sub-G_1 phase cells in this trial whereas HBL-100 was negative in both regards (Fig. 8*b*). We concluded that deficits of BIN1 in malignant breast cells were functionally significant and that ectopic re-expression of BIN1 caused programmed cell death, without regard to estrogen receptor status.

DISCUSSION

Our results demonstrate that loss of BIN1 in neoplastic breast disease is both frequent and significant. Partial or complete reductions in steady-state BIN1 levels were documented in greater than half of the cases of primary breast cancer and in all of the breast carcinoma cell lines examined. No correlation between

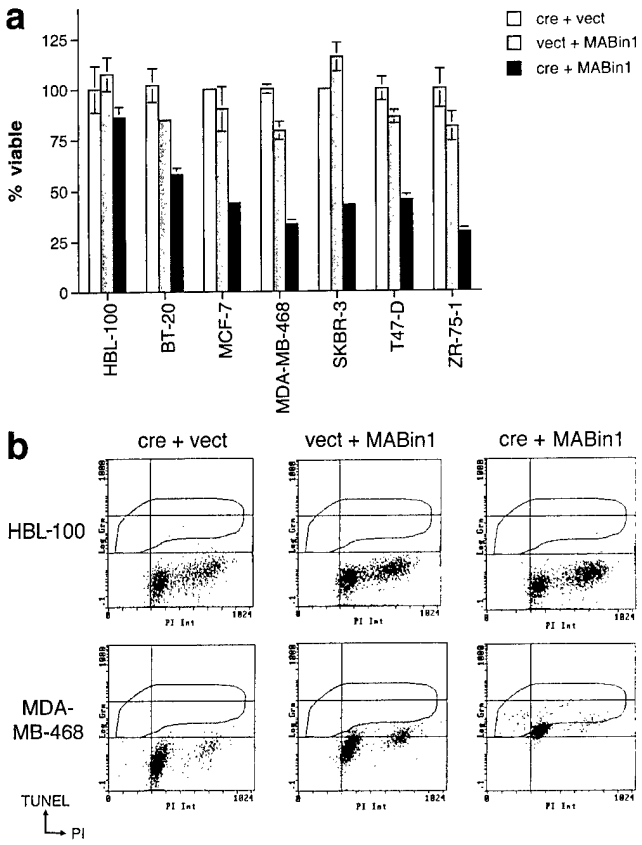


FIGURE 8 – BIN1 induces programmed cell death in malignant, but not benign, cells. *(a)* Cell viability. Cells were infected with the viruses indicated in Figure 3, and viable cells were counted by Trypan blue staining 48 hr later. The data represent the mean and standard error derived from 3 separate trials. *(b)* Flow cytometry. HBL-100 and MDA-MB-468 cells were infected as above and 48 hr later processed for TUNEL reaction and propidium iodide staining.

BIN1 status and other pathological markers was noted, though the sample size was small. The *BIN1* gene is located on chromosome 2q14 (Negorev *et al.*, 1996) within a region reported to be deleted in 30% to 40% of breast carcinomas (Kerangueven *et al.*, 1997). However, although we documented homozygous deletion of the *BIN1* gene in the tumor cell line SK-BR-5 and obtained some evidence of aberrant 5′ organization in malignant cell lines and primary tumors consistent with allelic deletions, further investigation is required to firmly establish the extent of genetic alterations of *BIN1* in breast cancer. Nevertheless, given a conservative interpretation, the frequency of BIN1 losses occuring at the level of protein and/or message appears to exceed the frequency of genetic alterations, suggesting a role for epigenetic alterations. The *BIN1* promoter contains a CpG island (Wechsler-Reya *et al.*, 1997*b*), so DNA methylation events that affect promoter activity offer a likely mechanism for epigenetic alteration. In any case, the losses in BIN1 found to occur frequently in breast cancer appeared to have both genetic and epigenetic causes.

TABLE II – PROPORTION OF BIN1-EXPRESSING CELLS EXHIBITING DNA DEGRADATION

Cell line	ER status	Adenoviral vectors		
		cre + vect	vect + MABIN1	cre + MABIN1
HBL-100	+	9.6	6.6	10.1
BT20	−	1.3	1.9	4.0
MDA-MB-468	−	1.8	3.6	23.6
SK-BR-3	−	2.7	2.9	19.7
T47D	+	2.9	4.8	9.1
ZR-75-1	+	5.5	5.7	20.0
MCF7	+	2.7	3.4	6.2

Cells were infected with 100 m.o.i. of each vector indicated and 48 hr later fixed, stained with propidium iodide and processed for flow cytometry. Results represent the proportion of cells in the population in the sub-G_1 fraction (mean of 2 trials). ER, estrogen receptor.

Evidence was obtained that BIN1 deficits were significant to the survival of malignant breast cells. Re-introduction of BIN1 into malignant cells which lacked endogenous BIN1 expression led to a selective induction of programmed cell death. This response, which did not occur in non-malignant cells, was consistent with other evidence supporting a tumor-suppressor role for this nucleocytoplasmic adaptor protein (Sakamuro *et al.*, 1996; Elliott *et al.*, 1999; Ge *et al.*, 1999). Although its functions as an adaptor appear complex, due in part to modulation by tissue-specific splicing, BIN1 was identified initially through its ability to interact with and inhibit the oncogenic properties of MYC. Activation of MYC occurs frequently in later stages of breast cancer, and this event is associated with poor prognosis (Berns *et al.*, 1992; Borg *et al.*, 1992; Watson *et al.*, 1993). Since BIN1 can block malignant transformation by MYC (Sakamuro *et al.*, 1996), the loss of BIN1 activity in breast cancer cells may promote full oncogenic activation of MYC by eliminating a key governor. The governor may act downstream rather than upstream of MYC: evidence supports a model in which BIN1 is necessary for MYC-mediated apoptosis such that loss of BIN1 may relieve the pro-apoptotic penalty of activated MYC (Prendergast, 1999). BIN1 can also inhibit neoplastic transformation caused by adenovirus E1A, human papillomavirus E7 or mutant p53 (Elliott *et al.*, 1999). Thus, it is also conceivable that loss of BIN1 may contribute to breast cancer progression in more than one way by eliminating a MYC-independent mechanism which can limit neoplastic transformation.

ACKNOWLEDGEMENTS

We are grateful to Dr. F.L. Graham for providing the cre-loxP adenoviral system, Dr. J.M. Wilson for providing the empty adenovirus vector Ad-vect and Dr. K. Satyamoorthy for advice on adenoviral infection. We also thank Mr. R. Buccafusca for expert technical assistance. Support from the Wistar Cancer Core grant (CA10851) and the Wistar Histology and Flow Cytometry Core Facilities is acknowledged. This work was supported by grants to GCP from the US Army Breast Cancer Research Program (DAMD17–96–1-6324) and the American Cancer Society (CN-160). KG is the recipient of a fellowship award from the Adler Foundation. GCP is the recipient of awards from CapCURE and is a Pew Scholar in the Biomedical Sciences.

REFERENCES

ANTON, M. and GRAHAM, F.L., Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J. Virol.*, **69,** 4600–4606 (1995).

BERNS, E.M., KLIJN, J.G., VAN PUTTEN, W.L., VAN STAVEREN, I.L., PORTENGEN, H. and FOEKENS, J.A., c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. *Cancer Res.*, **52,** 1107–1113 (1992).

BORG, A., BALDETORP, B., FERNO, M., OLSSON, H. and SIGURDSSON, H., c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. *Int. J. Cancer,* **51,** 687–691 (1992).

CHURCH, G.M. and GILBERT, W., Genomic sequencing. *Proc. nat. Acad. Sci. (Wash.),* **81,** 1991–1995 (1984).

DAVIS, A.R. and WILSON, J.M., Adenoviral vectors. *In: Current protocols in human genetics,* pp. 12.4.1–18, J. Wiley, New York (1996).

ELLIOTT, K., SAKAMURO, D., BASU, A., DU, W., WUNNER, W., STALLER, P., GAUBATZ, S., ZHANG, H., PROCHOWNIK, E., EILERS, M. and PRENDERGAST, G.C., BIN1 functionally interacts with Myc in cells and inhibits cell proliferation by multiple mechanisms. *Oncogene,* **18,** 3564–3573 (1999).

GE, K., DUHADAWAY, J., DU, W., HERLYN, M., RODECK, U. and PRENDERGAST, G.C., Novel mechanism for elimination of a tumor suppressor: aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. *Proc. nat. Acad. Sci. (Wash.),* **96,** 9689–9694 (1999).

HERRMANN, B.G. and FRISCHAUF, A.M., Isolation of genomic DNA. *Methods Enzymol.,* **152,** 180–183 (1987).

KERANGUEVEN, F., NOGUCHI, T., COULIER, F., ALLIONE, F., WARGNIEZ, V., SIMONY-LAFONTAINE, J., LONGY, M., JACQUEMIER, J., SOBOL, H., EISINGER, F. and BIRNBAUM, D., Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. *Cancer Res.,* **57,** 5469–5474 (1997).

NASS, S.J. and DICKSON, R.B., Defining a role for MYC in breast tumorigenesis. *Breast Cancer Res. Treat.,* **44,** 1–22 (1997).

NEGOREV, D., REITHMAN, H., WECHSLER-REYA, R., SAKAMURO, D., PRENDERGAST, G.C. and SIMON, D., The *BIN1* gene localizes to human chromosome 2q1.4 by PCR analysis of somatic cell hybrids and fluorescence *in situ* hybridization. *Genomics,* **33,** 329–331 (1996).

PRENDERGAST, G.C., Mechanisms of apoptosis by c-Myc. *Oncogene,* **18,** 2966–2986 (1999).

PRENDERGAST, G.C. and COLE, M.D., Posttranscriptional regulation of cellular gene expression by the *c-myc* oncogene. *Mol. cell. Biol.,* **9,** 123–134 (1989).

PRENDERGAST, G.C., LAWE, D. and ZIFF, E.B., Association of Myn, the murine homolog of Max, with MYC stimulates methylation-sensitive DNA binding and Ras cotransformation. *Cell,* **65,** 395–407 (1991).

SAKAMURO, D., ELLIOTT, K., WECHSLER-REYA, R. and PRENDERGAST, G.C., BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nature (Genet.),* **14,** 69–77 (1996).

SAKAMURO, D. and PRENDERGAST, G.C., New Myc-binding proteins: a second Myc network emerges. *Oncogene,* **18,** 2942–2953 (1999).

SCHWARTZ, L.M. and OSBORNE, B.A. (eds.), *Cell death,* Academic Press, New York (1995).

SHIOZAWA, M., HIRAOKA, Y., YASUDA, K., IMAMURA, T., SAKAMURO, D., TANIGUCHI, N., YAMAZOE, M. and YOSHIKAWA, H., Synthesis of human gamma-glutamyl transpeptidase (GGT) during the fetal development of liver. *Gene,* **87,** 299–303 (1990).

WATSON, P.H., SAFNECK, J.R., LE, K., DUBIK, D. and SHIU, R.P., Relationship of MYC amplification to progression of breast cancer from *in situ* to invasive tumor and lymph node metastasis. *J. nat. Cancer Inst.,* **85,** 902–907 (1993).

WECHSLER-REYA, R., ELLIOTT, K., HERLYN, M. and PRENDERGAST, G.C., The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. *Cancer Res.,* **57,** 3258–3263 (1997*a*).

WECHSLER-REYA, R., ELLIOTT, K. and PRENDERGAST, G.C., A role for the putative tumor suppressor BIN1 in muscle cell differentiation. *Mol. cell. Biol.,* **18,** 566–575 (1998).

WECHSLER-REYA, R., SAKAMURO, D., ZHANG, J., DUHADAWAY, J. and PRENDERGAST, G.C., Structural analysis of the human *BIN1* gene: evidence for tissue-specific transcriptional regulation and alternate RNA splicing. *J. biol. Chem.,* **272,** 31453–31458 (1997*b*).

Oncogene (2000) 19, 4669–4684

www.nature.com/onc

The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program

Katherine Elliott[1], Kai Ge[1,3], Wei Du[1,2] and George C Prendergast*[,1,2]

[1]*The Wistar Institute, Philadelphia, PA, USA;* [2]*Glenolden Laboratory, DuPont Pharmaceuticals Company, Glenolden, PA, USA*

Cell death processes are progressively inactivated during malignant development, in part by loss of tumor suppressors that can promote cell death. The Bin1 gene encodes a nucleocytosolic adaptor protein with tumor suppressor properties, initially identified through its ability to interact with and inhibit malignant transformation by c-Myc and other oncogenes. Bin1 is frequently missing or functionally inactivated in breast and prostate cancers and in melanoma. In this study, we show that Bin1 engages a caspase-independent cell death process similar to type II apoptosis, characterized by cell shrinkage, substratum detachment, vacuolated cytoplasm, and DNA degradation. Cell death induction was relieved by mutation of the BAR domain, a putative effector domain, or by a missplicing event that occurs in melanoma and inactivates suppressor activity. Cells in all phases of the cell cycle were susceptible to death and p53 and Rb were dispensable. Notably, Bin1 did not activate caspases and the broad spectrum caspase inhibitor ZVAD.fmk did not block cell death. Consistent with the lack of caspase involvement, dying cells lacked nucleosomal DNA cleavage and nuclear lamina degradation. Moreover, neither Bcl-2 or dominant inhibition of the Fas pathway had any effect. In previous work, we showed that Bin1 could not suppress cell transformation by SV40 large T antigen. Consistent with this finding, we observed that T antigen suppressed the death program engaged by Bin1. This observation was interesting in light of emerging evidence that T antigen has roles in cell immortalization and human cell transformation beyond Rb and p53 inactivation. In support of a link to c-Myc-induced death processes, AEBSF, a serine protease inhibitor that inhibits apoptosis by c-Myc, potently suppressed DNA degradation by Bin1. Our findings suggest that the tumor suppressor activity of Bin1 reflects engagement of a unique cell death program. We propose that loss of Bin1 may promote malignancy by blunting death penalties associated with oncogene activation. *Oncogene* (2000) **19**, 4669–4684.

Keywords: transformation; apoptosis; cancer; amphiphysin-like protein/amphl

Introduction

Cell suicide programs are crucial to development and homeostasis. A major role of these programs is to stanch inappropriate cell proliferation that can lead to cancer. Indeed, loss of the capacity for programmed cell death (PCD) is a hallmark of the malignant cell. Loss of this capacity is not due to inactivation of the machinery responsible for the major form of PCD, apoptosis, which is minimally comprised of cytochrome c, apoptosis promoting factors (Apafs), and caspases (Reed *et al.*, 1998). Instead, it appears that malignant cells suppress or eliminate signals needed to commit to PCD and/or to activate the apoptotic machinery.

How cells commit to die and how malignant cells sidestep this decision are questions of great interest in the areas of programmed cell death and cancer research. Caspase regulation is important, but there is emerging evidence that caspase-independent processes may also have important roles. Death receptors directly activate caspases and cancer cells neutralize these routes by multiple strategies (Ashkenazi and Dixit, 1998). Mitochondria indirectly regulate caspases, by controlling the release of cytochrome c release and thereby the status of Apaf, which controls the activation of caspase-9 (Green and Reed, 1998). This route is blunted in cancer cells primarily by alterations in the level of Bcl-2 family proteins, which control cytochrome c release (Chao and Korsmeyer, 1998; Reed *et al.*, 1998; Thompson and Vander Heiden, 1999), but probably at other levels as well (Ding *et al.*, 1998; Fearnhead *et al.*, 1997). Caspase activation is clearly sufficient for death commitment but whether it is necessary is much less clear. A viable hypothesis is that caspase-independent processes participate in commitment and that caspase activation seals a PCD decision made by the cell (Amarante-Mendes *et al.*, 1998; McCarthy *et al.*, 1997; Thompson and Vander Heiden, 1999). If so, then caspase-independent processes may be disrupted in cancer cells like caspase activation pathways.

One area of investigation into cancer cell death mechanisms centers on how c-Myc stimulates PCD and why it does not do so in malignancy (Prendergast, 1999). Oncogenic activation of c-Myc promotes the development of many clinically significant cancers, such as those of the breast, colon, lung, and prostate (Cole, 1986; Garte, 1993; Jenkins *et al.*, 1997). c-Myc activation usually occurs at later stages in carcinoma in humans and is usually a poor prognostic marker. However, in premalignant cells c-Myc is a robust stimulator of PCD. Therefore, to exploit the growth-promoting aspects of c-Myc, malignant cells must evolve strategies to escape the death penalty associated

*Correspondence: G C Prendergast, Glenolden Laboratory, DuPont Pharmaceuticals Co., Glenolden, PA, USA,
[3]Current address: The Rockefeller University, New York, NY, USA
Received 13 March 2000; revised 2 May 2000; accepted 10 May 2000

with its activation. Mechanistic investigations in fibroblast and lymphocyte models have defined central roles for the tumor suppressors p53 and p19^{ARF}, which are frequently inactivated in human cancer (Sherr, 1998). Whether these genes mediate PCD by c-Myc or sensitize cells to its action is unclear. Nevertheless, it is evident that p53 inactivation does not compromize the ability of c-Myc to drive PCD in epithelial cells (Prendergast, 1999; Sakamuro *et al.*, 1995), indicating that p53-independent mechanisms are also important. Interestingly, careful investigations have revealed that caspase inhibition or Bcl-2 overexpression does not abolish the ability of c-Myc to commit cells to undergo PCD. Caspase inactivation eliminates nuclear phenotypes characteristic of apoptosis and slows the kinetics of cell death, but it does not abolish all apoptotic phenotypes nor ultimate cellular demise (Amarante-Mendes *et al.*, 1998; Cecconi *et al.*, 1998; McCarthy *et al.*, 1997; Soengas *et al.*, 1999; Yoshida *et al.*, 1998). Similarly, Bcl-2 proteins significantly delay but do not abolish PCD commitment induced by c-Myc either *in vitro* or *in vivo* (McCarthy *et al.*, 1997; Trudel *et al.*, 1997; Tsuneoka and Mekada, 2000). Thus, while c-Myc promotes PCD by activating caspases (Kangas *et al.*, 1998), and Bcl-2 cooperates with c-Myc to promote malignancy by delaying this process, c-Myc apparently also affects caspase-independent processes that influence death commitment. Inactivation of such processes may be important in epithelial cells where c-Myc activation occurs. This may be especially true at stages when malignant cell division is slow and inefficient modes of cell death which do not involve caspases may be effective at stanching tumor outgrowth. Thus, inactivation of caspase-independent processes may contribute to tumorigenesis by helping cells escape death penalties associated with activation of c-Myc or other oncogenes (Prendergast, 1999).

In this study, we investigated a role in PCD for Bin1 (Bridging INtegrator-1), one of an emerging set of c-Myc-interacting adaptor proteins that are candidates for regulating c-Myc or mediating its diverse actions in cells (Sakamuro and Prendergast, 1999). Bin1 function is complex and varied by tissue-specific splicing. It was identified initially through its ability to interact with and inhibit malignant transformation by c-Myc (Sakamuro *et al.*, 1996). Subsequent investigations established that there are two ubiquitous slice isoforms of Bin1 and several other splice forms that are restricted in expression to muscle or brain (Butler *et al.*, 1997; Ramjaun *et al.*, 1997; Tsutsui *et al.*, 1997; Wechsler-Reya *et al.*, 1997b, 1998). Bin1 polypeptides are related in their terminal domains to amphiphysin, a neuronal protein involved in synaptic vesicle endocytosis, and brain isoforms which are most similar have been termed alternately amphiphysin II or amphiphysin-like (amphl). However, amphiphysin was named for its biochemical properties, rather than its function, and outside the brain Bin1 has functions that are not amphiphysin-like. First, although the brain isoforms of Bin1 are cytosolic, the ubiquitous Bin1 isoforms localize to the nucleus as well as to the cytosol (Kadlec and Pendergast, 1997; Wechsler-Reya *et al.*, 1997a). Consistent with their nuclear localization, these isoforms functionally interact with the nuclear tyrosine kinase c-Abl as well as with c-Myc (Elliott *et al.*, 1999; Kadlec and Pendergast, 1997). Second, only brain isoforms of Bin1 include sequences that are required to interact with clathrin and the endocytosis regulatory complex AP-2 (Ramjaun and McPherson, 1998). Third, muscle-specific isoform, which localizes to the nucleus and binds c-Myc, is required for myoblasts to withdraw from the cell cycle and to terminally differentiate (Mao *et al.*, 1999; Wechsler-Reya *et al.*, 1998). Lastly, isoforms that localize to the nucleus and bind c-Myc exhibit tumor suppressor properties which are inactivated or missing in malignant melanoma, breast cancer, and prostate cancer (Ge *et al.*, 1999, 2000a,b). In contrast, amphiphysin and brain isoforms of Bin1 lack suppressor activity. Indeed, one way by which Bin1 is functionally inactivated in cancer is by missplicing of one of its brain-specific exons (Ge *et al.*, 1999). Thus, Bin1 has two functions, one of which is linked to nuclear processes that influence cell fate. In this study, we provide evidence that the tumor suppressor properties of Bin1 are related to induction of cell death and that Bin1 participates in a caspase-independent process similar to type II apoptosis.

Results

Bin1 activates a programmed cell death process in malignant cells

We have shown that reintroduction of Bin1 into human cancer cell lines that lack endogenous expression leads to loss of proliferative capacity and cellular demise (Elliott *et al.*, 1999; Ge *et al.*, 1999, 2000a; Sakamuro *et al.*, 1996). To investigate this phenomenon in more detail, we used a set of recombinant adenoviral vectors that efficiently deliver Bin1 to human cells. One vector was constitutive and used the cytomegalovirus (CMV) early promoter to drive Bin1 expression (Ad-Bin1). A second vector was inducible and allowed Bin1 expression to be controlled by coexpression of Cre recombinase. In this vector, Bin1 cDNA was inserted downstream of a CMV promoter and a stuffer cassette flanked by loxP sites (Ad-MABin1). A matched control virus was also constructed for each system in which a loss-of-function Bin1 gene was expressed (see below). Recombinant viruses were generated in human 293 cells by standard methods. DNA sequencing from amplified viral DNA confirmed the expected structure of each transgene (data not shown). For control experiments, we also prepared adenoviruses expressing LacZ (Ad-LacZ) or Cre recombinase (Ad-cre) in 293 cells. The host cell line used for infection was HepG2 hepatoma cells, a functionally null cell which lacks endogenous Bin1 expression and is susceptible to Bin1-mediated growth suppression (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996).

Infections employing Ad-lacZ indicated that a multiplicity of infection (m.o.i.) of 50–100 was required to infect >90% of HepG2 cells exposed to virus *in vitro* (data not shown). HepG2 cells infected with Ad-Bin1 at 10, 50 or 100 m.o.i. exhibited increasing levels of Bin1 expression as documented by Western analysis of cell extracts prepared and analysed 48 h after infection (see Figure 1a). Bin1 was detectable within 12 h of infection and reached a maximum by 48 h (see Figure 1b). When infected under similar conditions, the level of ectopic Bin1 in IMR90 diploid

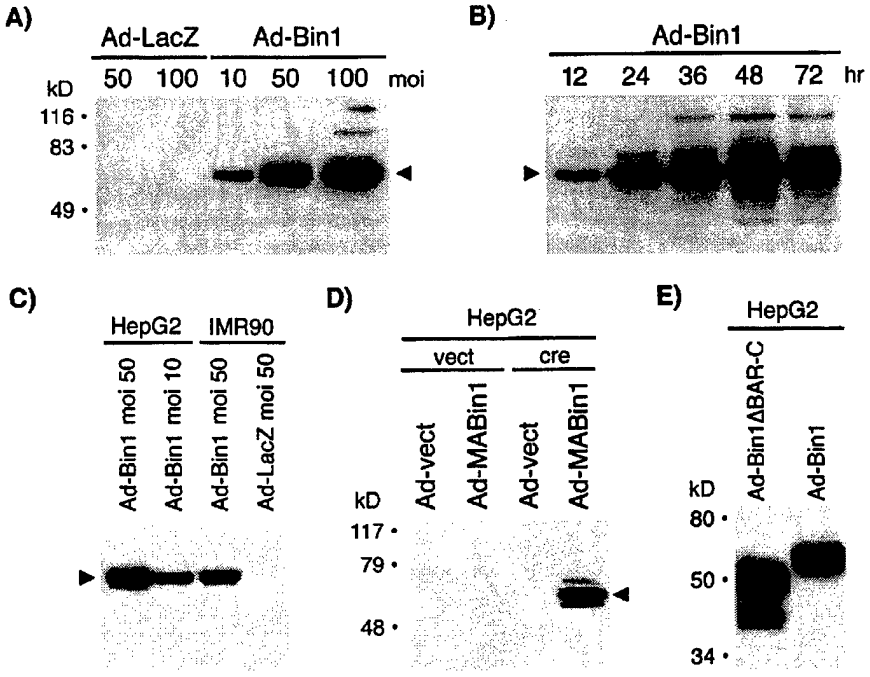


Figure 1 Recombinant Bin1 adenoviruses. Western analysis of whole cell lysates prepared from cells infected with the adenoviral vectors indicated was performed. The anti-Bin1 monoclonal antibodies 99D were used as the primary antibodies in panels a, b, and d; antibodies 99D plus 99I were used in panel c (Wechsler-Reya *et al.*, 1997a). (**a**) Expression from the constitutive vector Ad-Bin1. HepG2 cells lacking endogenous Bin1 were incubated with viruses at the multiplicity of infection (m.o.i.) indicated and extracts were prepared 48 h later. (**b**) Ad-Bin1 time course. Extracts were prepared from HepG2 cells harvested at the times indicated. (**c**) Comparative levels of expression in IMR90 diploid fibroblasts or HepG2 cells. Extracts were prepared 48 h after infection at the indicated m.o.i. IMR90 express endogenous Bin1 (Sakamuro *et al.*, 1996) but at a level that is undetectable on this exposure of the blot, which illustrates similar steady-state levels of Bin1 in IMR90 or HepG2 cells infected with the same amount of Ad-Bin1. (**d**) Cre-inducible expression from Ad-MABin1. HepG2 cell lines stably expressing P1 bacteriophage Cre site-specific recombinase (HepG2/cre) or containing only vector sequences (HepG2/CMV) were incubated with the virus indicated (m.o.i. = 100) and extracts were prepared 48 h later. The transgene in Ad-MABin1 is located downstream of a loxP site-flanked stuffer sequence such that expression occurs only after Cre-induced recombination (see Materials and methods). Ad-vect is a control virus that contain no transgene. (**e**) Expression of the loss-of-function deletion mutant Bin1ΔBAR-C. Western analysis was performed using extracts isolated from cells infected with 100 m.o.i. Ad-Bin1 or Ad-Bin1ΔBAR-C, which lacks anti-transforming and tumor suppressor properties (Elliott *et al.*, 1999b)

fibroblasts used in control experiments was comparable to that seen in HepG2 cells (see Figure 1c). Indirect cell immunofluorescence experiments indicated that the high level of expression driven by Ad-Bin1 was correlated with localization of Bin1 throughout the cell. To document Bin1 expression from the Cre-inducible vector Ad-MABin1, we infected HepG2 cell lines that stably expressed Cre recombinase (HepG2/cre) or that contained only vector sequences (HepG2/CMV). Bin1 was detected in HepG2/cre cells infected with 50 m.o.i. of Ad-MABin1 (see Figure 1d). In contrast, Bin1 was not detected after infection of HepG2/CMV cells, where the stuffer upstream of Bin1 could not be removed, nor was it detected in either cell line infected with control viruses that lacked an insert (Ad-vect). A constitutive adenoviral vector was also constructed for a loss-of-function mutant of Bin1, termed Bin1ΔBAR-C, that lacks a.a. 125–206 within the N-terminal BAR domain which is crucial for antineoplastic activity (Elliott *et al.*, 1999). Extracts derived from cells infected with Ad-Bin1ΔBAR-C exhibited a polypeptide with the expected apparent mobility of ~50 kD (see Figure 1e). A second smaller polypeptide was also detected at lower levels relative to the Bin1ΔBAR-C protein on Western blots. The appearance of this polypeptide did not affect the loss-of-function of Bin1ΔBAR-C, which was employed as a negative control for cell death induction (see below). In summary, these experiments validated the adenoviral vectors for constitutive or inducible Bin1 expression in human cells.

HepG2 cells expressing Bin1 displayed striking morphological changes. Cells assumed a rounded, shrunken morphology and exhibited deformations of the plasma membrane before completely detaching from substratum (see Figure 2a). The changes seen were consistent with induction of PCD like that observed previously (Ge *et al.*, 1999, 2000a). Cells infected with Ad-lacZ at similar or higher m.o.i. did not display similar morphologies. No signs of cellular demise were noted following infections of normal human diploid IMR90 fibroblasts with Ad-Bin1 (see Figure 2a). IMR90 cells showed some enlargement but flow cytometry indicated no signs of cell cycle arrest (data not shown), consistent with previous observations in rodent embryo fibroblasts, Rat1 fibroblasts, or mouse C2C12 myoblasts (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1998; unpublished observations). Thus, Bin1 was not grossly toxic to cells, consistent with previous observations (Ge *et al.*, 1999, 2000a;

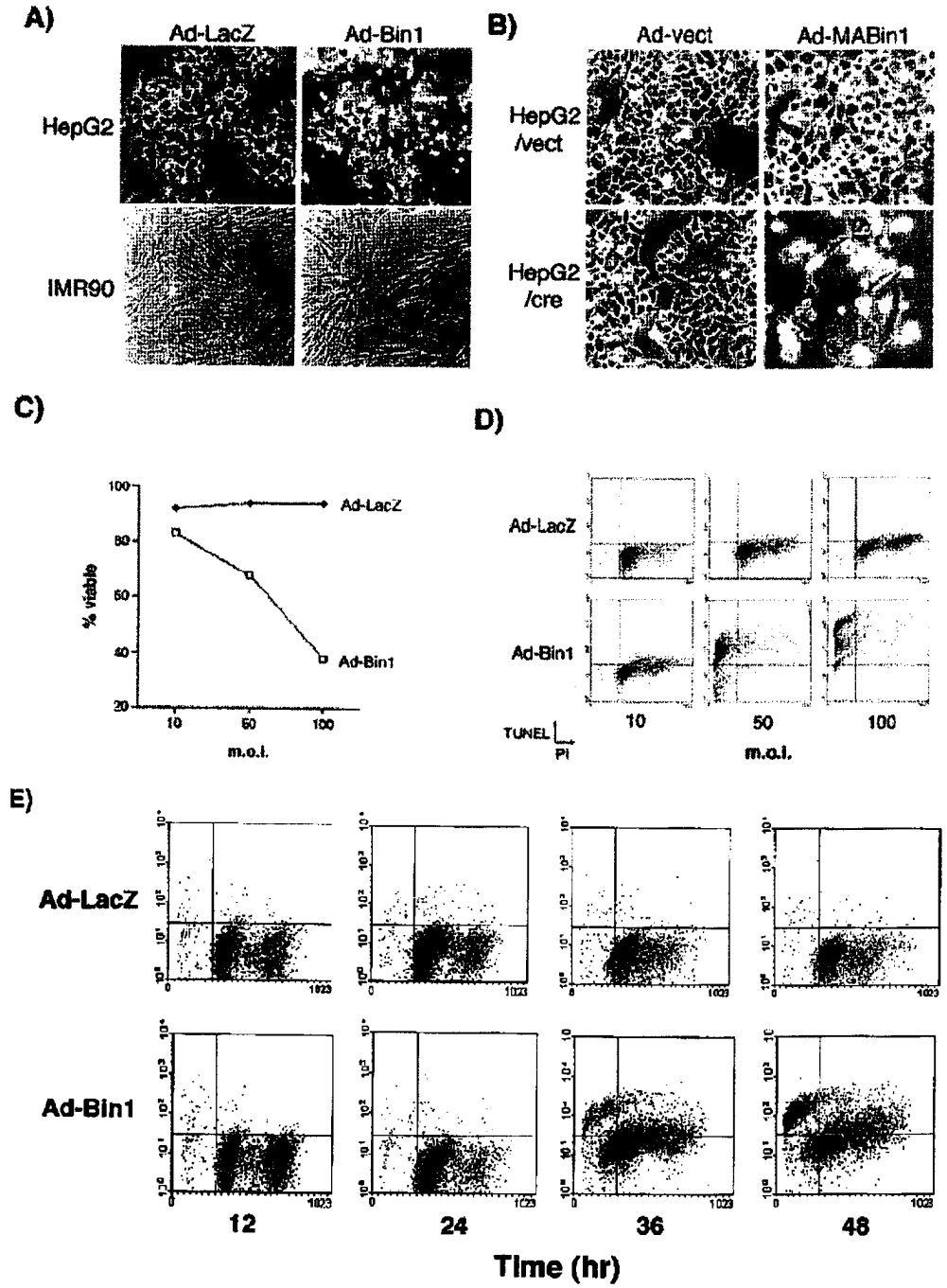


Figure 2 Bin1 induces demise of malignant cells. (**a**) Morphology of HepG2 hepatoma cells or IMR90 diploid fibroblasts following infection with Ad-LacZ or Ad-Bin1. Cells were incubated with 100 m.o.i. virus indicated and photographed 48 h later using phase-contrast optics (magnification = 100×). (**b**) Morphology of cells infected with inducible Ad-MABin1 virus. HepG2 cells stably expressing Cre recombinase or vector only were incubated with 100 m.o.i. virus indicated and processed as above. (**c**) Viable cell count. Cells were infected with the viruses indicated, as in Figure 1, and harvested by trypsinization 48 h later. The proportion of viable cells in the population was determined by trypan blue exclusion. (**d**) Cell death is associated with DNA degradation and occurs throughout the cell cycle. Cells were incubated with the indicated m.o.i. of Ad-LacZ or Ad-Bin1 and processed 48 h later for TUNEL reaction, propidium iodide staining, and flow cytometry. In each graph, the X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity. Time courses indicated a correlation between the kinetics of the cytopathic effect and DNA degradation. (**e**) Cell death elicited by Bin1 occurs throughout the cell cycle. Cells were incubated with 50 m.o.i. Ad-LacZ or Ad-Bin1 and processed after the period indicated for TUNEL reaction, propidium iodide staining, and flow cytometry. In each graph, the X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity

Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1998). Induction of the cre-inducible virus Ad-MABin1 produced identical effects. In HepG2/CMV cells, the control virus Ad-vect had little morphological effect, whereas uninduced Ad-MABin1 had a slight effect on cell shape (see Figure 2b). The effect of the latter virus might reflect low leaky expression seen in other cell types infected by Ad-MABin1 (Ge *et al.*, 1999). However, the uninduced luciferase vector Ad-MA19 produced similar effects and we observed no increase in detached cells using either vector (data not shown), arguing for a nonspecific effect of the Ad-MA vector in HepG2 cells. In any case, there was a profound difference seen in HepG2/cre cells, where Ad-vect had no discernible effect but Ad-MABin1 elicited a dramatic increase in detached cells (see Figure 2b). Taken together, these results confirmed that the cytopathic effect elicited by the constitutive Ad-Bin1 vector was not due to nonspecific toxicity of the vector system.

To explicitly assess cell viability in infected cultures, cells incubated with various m.o.i. of Ad-Bin1 or Ad-lacZ were harvested 48 h after infection and the proportion of viable cells was determined by trypan blue exclusion. Cells infected with Ad-lacZ at all m.o.i. were >90% viable whereas cells infected with Ad-Bin1 exhibited a linear relationship between m.o.i. and loss of viability (see Figure 2c). Similar results were obtained using Ad-MABin1 (data not shown). Thus, the cytopathic phenotype elicited by Bin1 was associated with cell death.

We next examined whether cells induced to die by Bin1 exhibited any differences in endocytosis, because brain-specific splice isoforms of Bin1 that localize to the cytosol in differentiated neurons have been implicated in this process, like the related adaptor protein amphiphysin (David *et al.*, 1996; Ramjaun *et al.*, 1997; Wigge *et al.*, 1997). Ubiquitous splice isoforms of Bin1 that localize to the nucleus lack brain-specific exons required for interaction with clathrin-coated endocytotic vesicles and AP2 (Ramjaun and McPherson, 1998; P. deCamilli, personal communication). However, while this structural difference suggested strongly that endocytotic roles were probably brain-specific, we wished to explicitly rule out a role for endocytosis in cell death induction by the nonneuronal Bin1 isoform used in these experiments. Fluid-phase or receptor-mediated endocytosis was monitored by comparing the rate of uptake of horseradish peroxidase or fluorescein-conjugated transferrin, respectively (Barbieri *et al.*, 1998; Benmerah *et al.*, 1998). No differences were apparent in the level of uptake of either reagent in cells infected with Ad-Bin1 or Ad-LacZ (data not shown). These observations supported the expectation that the endocytotic function of the Bin1 gene is regulated by alternate splicing and that splice isoforms that localize to the nucleus do not have these roles (Kadlec and Prendergast, 1997; Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a, 1998). We concluded that the ability of Bin1 to induce cell death was unrelated to effects on endocytosis.

To begin to define the basis for cell death, we analysed HepG2 cells by flow cytometry following terminal transferase-catalyzed dUTP labeling of nicked DNA ends (TUNEL assay). Cells were infected with increasing m.o.i. of adenoviral vector and harvested for propidium iodide staining, TUNEL assay, and flow cytometry 48 h later. A dose-dependent increase in the proportion of TUNEL-positive cells was observed after Ad-Bin1 infection (see Figure 2d). TUNEL positivity was detected in cells in all phases of the cell cycle as indicating by propidium iodide staining. An increase in cells with sub-G1 phase DNA occurred in the TUNEL-positive population with similar kinetics. A time-course experiment confirmed observations that the appearance of TUNEL-positive cells and the accumulation of cells with sub-G1 phase DNA began 24–36 h after infection, corresponding to a time approximately 12–24 h after Bin1 expression (see Figure 2e). This experiment also highlighted the finding that apoptotic cells (as defined by TUNEL positivity) emerged from all phases of the cell cycle. Taken together, these results suggested that Bin1 induced a programmed cell death (PCD) process. Similar experiments performed with the inducible vector confirmed these observations and confirmed that they were dependent on Bin1 expression rather than a nonspecific effect of the adenoviral vector (data not shown). The features of DNA degradation seen in flow cytometry experiments paralleled the kinetics of morphological features of apoptosis and loss of viability. In multiple trials using each vector system, no reproducible effects of Bin1 on the distribution of cells in the cell cycle were noted, consistent with the observation that TUNEL-positive cells emerged from all phases. Taken together, the results suggested that Bin1 engaged a PCD process that could be initiated at any point in the cell cycle, similar to c-Myc and other oncogenes (Evan *et al.*, 1992).

BAR domain is crucial for Bin1 to induce cell death

The Bin1 BAR domain is crucial to inhibit transformation of rodent fibroblasts by c-Myc and to suppress the proliferation of HepG2 cells (Elliott *et al.*, 1999b). To determine whether this domain was also crucial for death activity, we infected cells with Ad-Bin1ΔBAR-C, which expresses the loss-of-function mutant Bin1Δ BAR-C (Elliott *et al.*, 1999). Ad-Bin1ΔBAR-C did not elicit the cell detachment and cytopathic phenotype produced by Ad-Bin1 (see Figure 3a). Similarly, flow cytometric analysis of cells harvested 48 h after viral infection showed that Ad-Bin1ΔBAR-C did not elicit DNA degradation, even at elevated multiplicities of infection (see Figure 3b). These observations provided additional evidence that the cytotoxic effects of Bin1 in HepG2 cells were not due to some nonspecific cause, since overexpression of a loss-of-function mutation that eliminated tumor suppressor properties also abolished PCD activity. We concluded that Bin1 engaged a PCD process that could explain the basis for its tumor suppressor properties.

p53 and Rb are dispensable for PCD by Bin1

p53 has a central role in many types of PCD but it is mutated in many cancers. The retinoblastoma protein (Rb) has been reported to be antiapoptotic in many systems but in some cases it also has proapoptotic roles (Bowen *et al.*, 1998; Day *et al.*, 1997). The central importance of p53 and Rb in PCD control prompted us to determine whether these genes were needed for Bin1 action. HepG2 cells have wild-type p53 and Rb

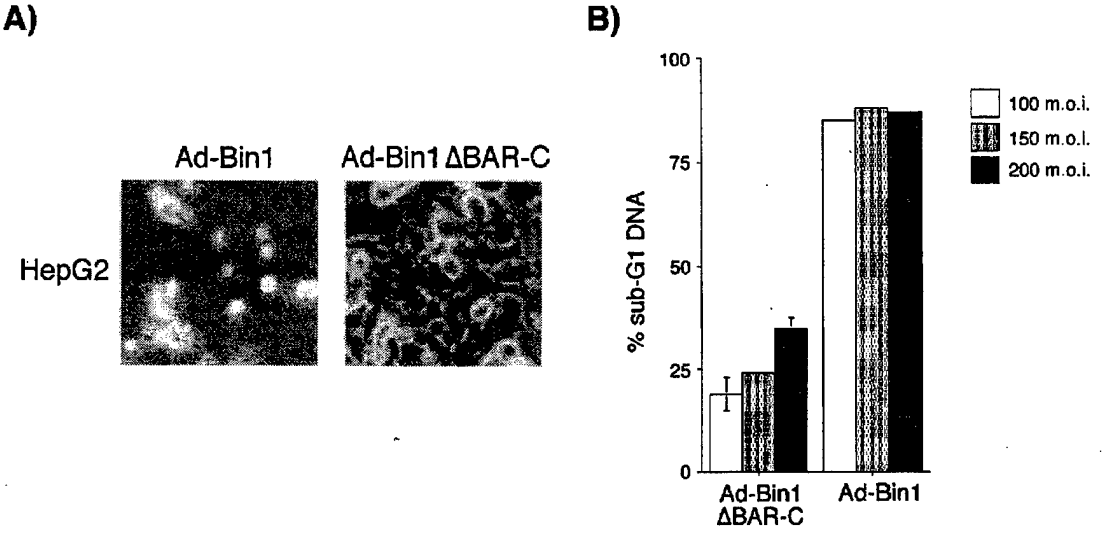


Figure 3 BAR deletion abolishes cell death by Bin1. (**a**) Lack of cytopathic effect. HepG2 cells were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 and photographed 48 h later (magnification = 100×). (**b**) Flow cytometry. Cells were infected with the m.o.i. virus indicated and harvested and processed 48 h after infection for flow cytometry

genes, so we examined the effect of Ad-Bin1 on another tumor cell line, SAOS-2 osteosarcoma, which has homozygous deletions in both genes. SAOS-2 cells were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 virus and expression was confirmed by β-galactosidase staining or Western blotting, respectively (data not shown). Parallel dishes of cells were examined for the appearance of morphological features of apoptosis or harvested and processed for TUNEL labeling and flow cytometry. Similar to its effects on HepG2, Ad-Bin1 caused cell rounding, plasma membrane deformations, and substratum detachment (Figure 4a). Flow cytometry showed an increase in the number of TUNEL-positive cells with sub-G1 phase DNA following Ad-Bin1 infection. Positive cells emerged from all phases of the cell cycle and kinetics were similar to those observed in HepG2 cells (see Figure 4b). These results were consistent with evidence that p53 is dispensable for PCD by c-Myc in epithelial cells (Sakamuro *et al.*, 1995; Trudel *et al.*, 1997) and with findings in breast cancer and melanoma cell lines that p53 status was not correlated with Bin1 susceptibility (Ge *et al.*, 1999, 2000a). Consistent with the SAOS-2 susceptibility, the results of extensive RNAse protection and Western analyses of HepG2 cells expressing Bin1 did not reveal any differences in the expression of a variety of cell cycle and apoptosis regulators, including p53, Rb family members, cell cycle-dependent kinase inhibitors (p16INK4, p14ARF, p21WAF1, p27KIP1, or p57KIP2), or Bcl-2 family genes (Bcl-2, Bcl-X_L, Mcl-1, Bik, Bax, or Bak). We concluded that Bin1 acted independently of the p53 and Rb pathways.

Bin1 does not activate caspases

The type of PCD process elicited by Bin1 was further characterized by examination of nuclear phenotypes associated with caspase activation. HepG2 cells infected with Ad-Bin1 but not Ad-LacZ displayed signs of nuclear deformation and chromatin margination at the nuclear periphery and at focal sites in the nucleoplasm (see Figure 5a). However, cells maintained considerable genomic DNA integrity and there was no evidence of nuclear lamina breakdown. This phenotype was different from that seen in classical or type I apoptosis, which is associated with a distinct nuclear condensation phenotype, and suggestive of type II apoptosis in which cytosolic features predominate. The contrast in nuclear morphology was highlighted by comparison to the nuclear phenotype elicited by the protein kinase inhibitor staurosporine, which induces type I apoptosis (see Figure 5a, right panel). Transient transfection of HepG2 cells with Bin1 plasmid vectors elicited the same nuclear phenotype as Ad-Bin1, ruling out a nonspecific adenovirus vector artifact (data not shown). To determine whether Bin1 caused nucleosomal DNA cleavage ('DNA laddering'), genomic DNA was isolated from cells infected with adenoviral vectors or treated with staurosporine and fractionated by agarose gel electrophoresis. Genomic DNA degradation was observed in cells infected by Ad-Bin1 but not by Ad-lacZ (see Figure 5b, left panel). This result was consistent with the ability of Bin1 to induce positive TUNEL reaction. However, Bin1 did not induce nucleosomal DNA cleavage, even though this feature could be induced in HepG2 cells by staurosporine (see Figure 5b, right panel).

The caspase-3 activated nuclease DFF/CAD is primarily responsible for nucleosomal DNA degradation and chromatin collapse, which is a characteristic of classical or type I apoptosis (Enari *et al.*, 1998; Liu *et al.*, 1997; Woo *et al.*, 1998). The absence of these features suggested that Bin1 did not activate caspase-3. To confirm this likelihood, caspase-3-like activities were assayed in extracts from Ad-Bin1-infected cells by measuring cleavage of the fluorescent substrate Ac-DEVD-pNA (see Figure 5c). Cleavage of Ac-DEVD-pNA by caspase-3-like activity was monitored by absorbance at 405 nM measured at various times after addition to extracts. Exogenous recombinant caspase-3 was used as a positive control for the assay. As expected, staurosporine induced caspase-3-like activity

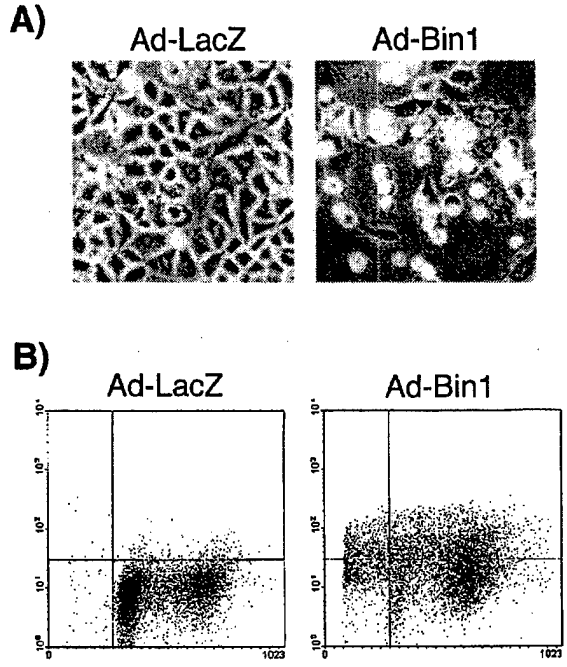


Figure 4 p53 and Rb are dispensable for programmed cell death by Bin1. SAOS-2 osteosarcoma cells, which have homozygous deletions of p53 and Rb, were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1. (**a**) Morphology. Cells were photographed 48 h after infection (magnification = 100 ×). (**b**) Flow cytometry. Cells were harvested 48 h after infection and processed for TUNEL assay and propidium iodide staining. Representative results from analysis by flow cytometry are shown. The X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity

in cell extracts. This induction confirmed that HepG2 cells expressed pro-caspase-3-like enzymes that were competent for activation by apoptotic stimuli, in support of evidence of a predominent role for caspase-3 in classical apoptotic responses in HepG2 cells (Suzuki *et al.*, 1998). In contrast, no significant cleavage of Ac-DEVD-pNA occurred in extracts prepared from cells infected with Ad-Bin1 or Ad-LacZ. In Western blotting experiments, we also did not detect proteolytic cleavage of either pro-caspase-3 or the caspase-3 substrate PARP (data not shown).

One possibility was that Bin1 activated a caspase other than caspase-3. To assess this possibility, we tested whether Bin1-induced cell death was suppressed by ZVAD.fmk, a broad spectrum inhibitor of caspases. In these experiments, the relative number of cells displaying sub-G1 phase DNA by flow cytometry was used as a measurement of PCD. As expected, ZVAD.fmk significantly blocked staurosporine-induced cell death. In contrast, ZVAD.fmk did not affect PCD by Bin1 even when added at high concentrations (see Figure 5d). Taken together, these results argued that the death process activated by Bin1 was caspase-independent.

To confirm this conclusion and gain additional insight into this death process we examined cells by electron microscopy. These experiments revealed cytosolic features consistent with a programmatic death process such as type II apoptosis (see Figure 6). Ad-lacZ-infected cells showed no signs of cytopathology. In contrast, staurosporine-treated cells were shrunken and exhibited cytosolic vacuolation, chromatin condensation, and nuclear degeneration (plasma membrane blebs were also observed but were not so dramatic in this cell system). Ad-Bin1-infected cells were similarly shrunken and heavily vacuolated. Margination of chromatin at the nuclear periphery was evident. However, there was no nuclear degeneration and limited chromatin condensation by comparison to staurosporine-treated cells. Cell nuclei were shrunken relative to Ad-LacZ-infected cells but not so severely as staurosporine-treated cells. Strikingly, the nuclear lamina in Ad-Bin1-infected cells remained essentially intact. This feature supported the lack of caspase involvement because lamins are subjected to caspase-mediated proteolysis during PCD (Lazebnik *et al.*, 1993; Rao *et al.*, 1996). In addition, lamin cleavage is separable from chromatin collapse (Lazebnik *et al.*, 1995) but is important for complete nuclear degeneration (Rao *et al.*, 1996), so the more limited nuclear degeneration in Ad-Bin1-infected cells was also consistent with a caspase-independent process. We observed no 'exploded' cells or flocculent densities in organelles or the cytosol that would signal necrosis. Trials in which ZVAD.fmk was added confirmed that the PCD phenotype induced by Bin1 was not affected by caspase inhibition. Addition of 100 μM ZVAD.fmk reduced cell volume and induced the appearance of focal densities in the nucleus, but these features were a nonspecific artifact of ZVAD.fmk, because the same features were also apparent in Ad-LacZ-infected cells. Interestingly, ZVAD.fmk did not block the Bin1 phenotype, in which cytosolic vacuolization predominated. In contrast, ZVAD.fmk dramatically affected the phenotype of staurosporine-treated cells, reversing nuclear degradation to a large extent. We concluded that Bin1 engaged a PCD process that was caspase-independent yet associated with limited chromatin degradation and cytosolic features of PCD.

Cell death by Bin1 is not blocked by Bcl-2 or Fas pathway inhibition

To further delineate the death process activated by Bin1 we investigated links to two classical PCD regulatory pathways. Bcl-2 proteins inhibit caspase activation through their ability to influence mitochondrial physiology, whereas death receptors directly activate caspases (Ashkenazi and Dixit, 1998; Thompson and Vander Heiden, 1999). HepG2 cell lines overexpressing Bcl-2 or a dominant inhibitor of FADD, which blocks Fas signals (Muzio *et al.*, 1996), were tested for response to Ad-Bin1. One would predict the response would be unaffected by Bcl-2 or Fas pathway disruption if Bin1 acted in a caspase-independent manner. Cells overexpressing Bcl-2 were resistant to staurosporine-induced apoptosis relative to vector control cells (data not shown). In contrast, there was no difference in the susceptibility of cells overexpressing Bcl-2 or the FADD dominant inhibitor to death induction by Bin1 (see Figure 7a). On a different line of work, we had observed that recombinant Bin1 baculoviruses increased the kinetics of cell death in Sf9 cells, and that Bcl-2 could not inhibit this effect (see Figure 7b), despite the fact that Bcl-2 inhibits baculovirus-induced cell death in this

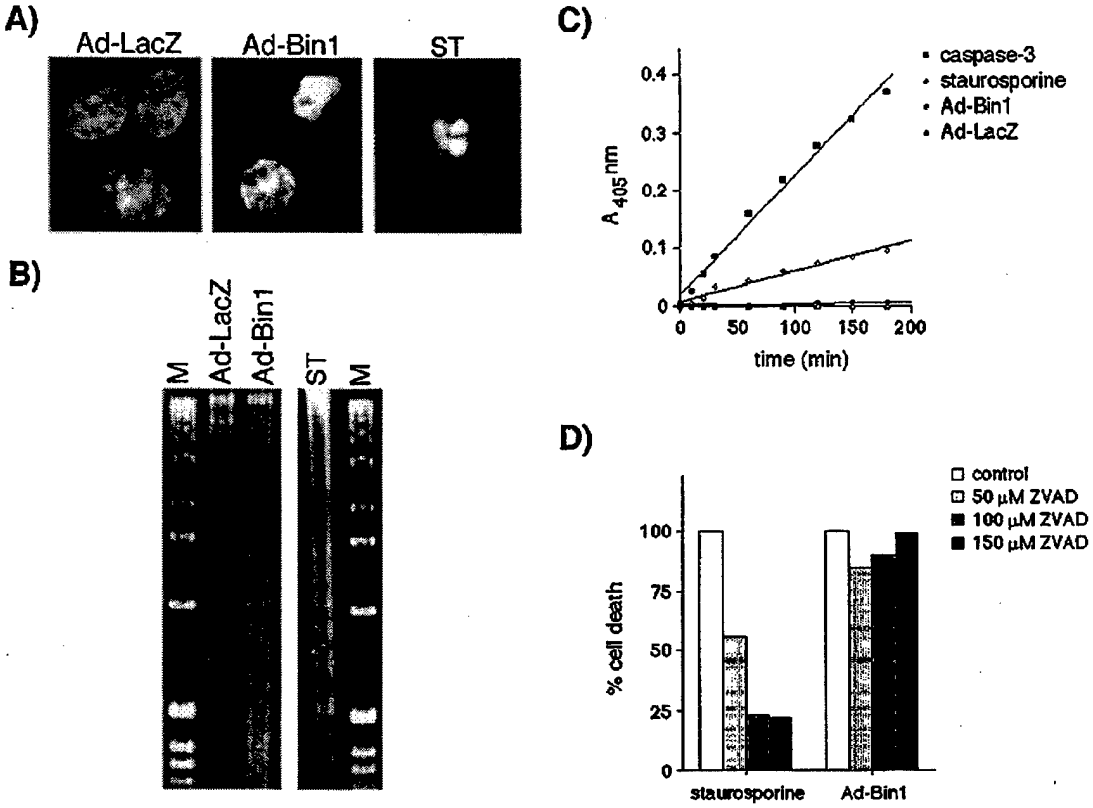


Figure 5 Lack of caspase activation or requirement. (**a**) Hoechst nuclear stain. HepG2 cells seeded on cover slips were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 and 48 h later fixed and stained with Hoechst dye, mounted, and examined by immunofluorescence microscopy. A separate culture was treated with 0.5 μM staurosporine as a positive control for apoptotic morphology. Cells were photographed at 400× magnification. (**b**) Nucleosomal DNA degradation. HepG2 cells were infected with viruses or treated with staurosporine as above. Hirt DNA (left panel) or total genomic DNA (right panel) was prepared and analysed by agarose gel electrophoresis. (**c**) Caspase-3 assay. HepG2 cells were infected with m.o.i. 100 Ad-LacZ or Ad-Bin1 or treated with 0.5 μM staurosporine and harvested 36 h later. Extracts were prepared and assayed for the presence of caspase-3-like activity using the colormetric substrate Ac-DEVD-pNA. Reactions were monitored for production of cleavage product at 405 nm at the times indicated. Recombinant caspase-3 was used as a positive control. (**d**) Caspase inhibition does not block Bin1-induced cell death. HepG2 cells were infected with 100 m.o.i. Ad-Bin1 or treated with 0.5 μM staurosporine in the presence or absence of the caspase inhibitor ZVAD.fmk at the concentration indicated. Cells were harvested 36 h later and processed for flow cytometry. The graph shows the relative proportion of the cell population undergoing cell death, using the appearance of sub-G1 phase DNA as an indicator

system (Alnemri *et al.*, 1992). In support of these results, we also obtained a set of negative results from experiments aimed at determining whether Bin1 caused cytochrome c release or altered mitochondrial membrane potential (data not shown). In summary, we concluded that Bin1 acted via non-classical mechanisms that were independent of the Bcl-2 and Fas pathways, two chief regulators of caspases in cells.

SV40 T antigen inhibits induction of cell death by Bin1

In previous work, we found that Bin1 inhibited Ras co-transformation of primary rodent fibroblasts by c-Myc and adenovirus E1A but not by SV40 large T antigen (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996). Since Bin1 activated a PCD process in malignantly transformed human cells we hypothesized that T antigen might suppress these effects. This hypothesis was not invalidated by the finding that p53 and Rb were dispensable for Bin1-induced death, because T antigen has additional role(s) in immortalization and human cell transformation beyond inactivation of these tumor suppressors (Conzen and Cole, 1995; Hahn *et al.*, 1999; Powell *et al.*, 1999). The effects of T antigen were examined using WI-38 diploid fibroblasts and a WI-38 derivative that expresses T antigen (WI-38/T cells). Like HepG2, WI-38 cells are functionally null for Bin1, in this case due to a missplicing event which causes loss-of-function identical to that which occurs in Bin1 in melanoma (Ge *et al.*, 1999). Western analysis confirmed RT–PCR results (Wechsler-Reya *et al.*, 1997b) establishing missplicing of brain-specific exon 12A in WI-38 cells and also in the WI-38/T derivative. The 12A isoform exhibited reduced mobility on SDS gels relative to wild-type Bin1 (see Figure 8a, top panel) and was also recognized by a monoclonal antibody that is specific for exon 12A-encoded residues (Ge *et al.*, 1999) (see Figure 8a, bottom panel).

T antigen blocked susceptibility to cell death by Bin1 (see Figure 8b,c). Loss of viability occurred only if Ad-MABin1 was induced by coinfection with Ad-cre virus. Death correlated with the appearance of rounded, detached cells similar to the cytopathic seen

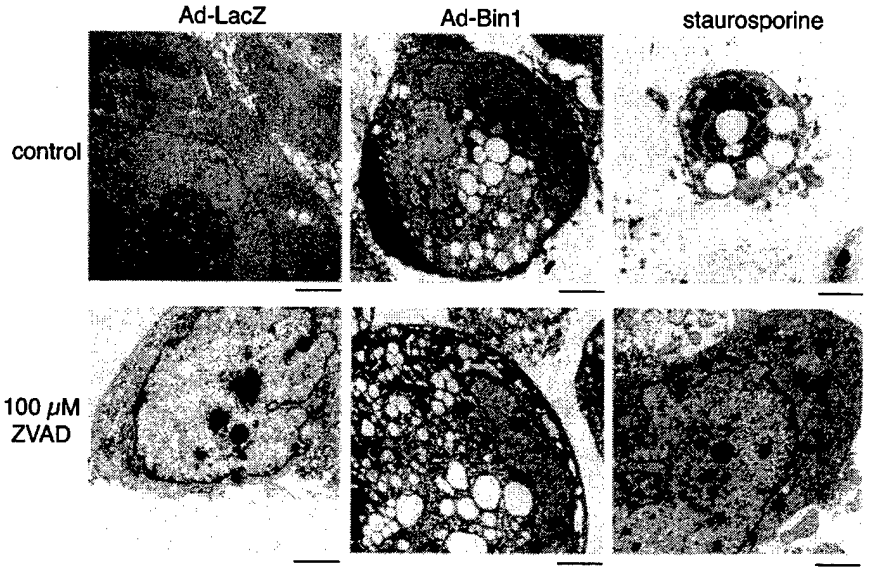


Figure 6 Death phenotype. HepG2 cells were infected with m.o.i. 200 Ad-LacZ or Ad-Bin1 or treated with 0.5 μM staurosporine as above, stained with osmium tetroxide, and processed for electron microscopy using standard methods. Where indicated, cells were treated with 100 μM ZVAD.fmk. The bars under each panel represent 2 μm

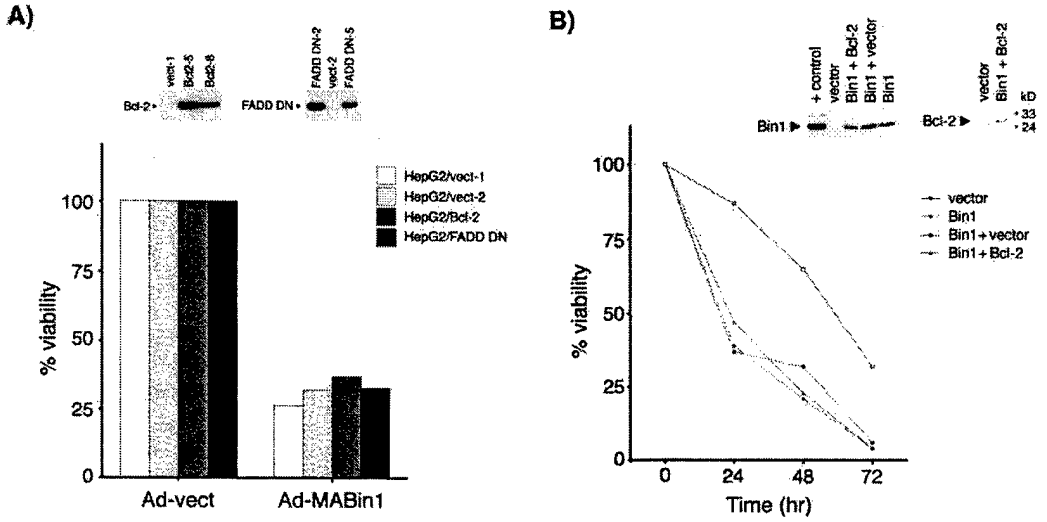


Figure 7 PCD by Bin1 is not inhibited by Bcl-2 or Fas pathway inhibition. (**a**) Bcl-2 or Fas pathway inhibition does not block Bin1-induced cell death. HepG2 cells overexpressing Bcl-2 or a dominant inhibitory mutant of the Fas-interacting adaptor protein FADD (Muzio *et al.*, 1996) were infected with 100 m.o.i. Ad-vect or Ad-MABin1 plus 100 m.o.i. Ad-cre and viability was measured by trypan blue exclusion 48 h later. Inset: Western analysis showing expression of Bcl-2 and FADD dominant negative (FADD DN) proteins relative to vector controls for each expression construct. (**b**) Increased kinetics of insect cell death elicited by recombinant Bin1 baculovirus are unaffected by Bcl-2. Sf9 cells were infected as described (Alnemri *et al.*, 1992; Elliott *et al.*, 1999b) and the proportion of viable cells in the culture were determined at various times post-infection. Inset: Western analysis of cell extracts processed at 24 h after infection demonstrating expression of Bin1 and Bcl-2

in HepG2 and other malignant cell lines (Ge *et al.*, 1999, 2000a). This response confirmed that the missplicing and inactivation of Bin1 in WI-38 was functionally meaningful. Similar results were obtained using derivatives of HepG2 that were engineered to express T antigen gave (data not shown). To confirm that endogenous misspliced isoform in WI-38 was truly a loss-of-function alteration, we infected cells with an adenoviral vector expressing this isoform (Ad-MABin1-10+12A) (Ge *et al.*, 1999). No loss of viability or cell detachment was observed. We concluded that SV40 T antigen suppressed PCD by Bin1.

A serine protease implicated in PCD by c-Myc inhibits DNA degradation by Bin1

The serine protease inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) has been reported to inhibit c-Myc-induced death in Rat1 fibroblasts (Kagaya *et al.*, 1997). This report also indicated that AEBSF does not block apoptosis induced by Fas activation, the

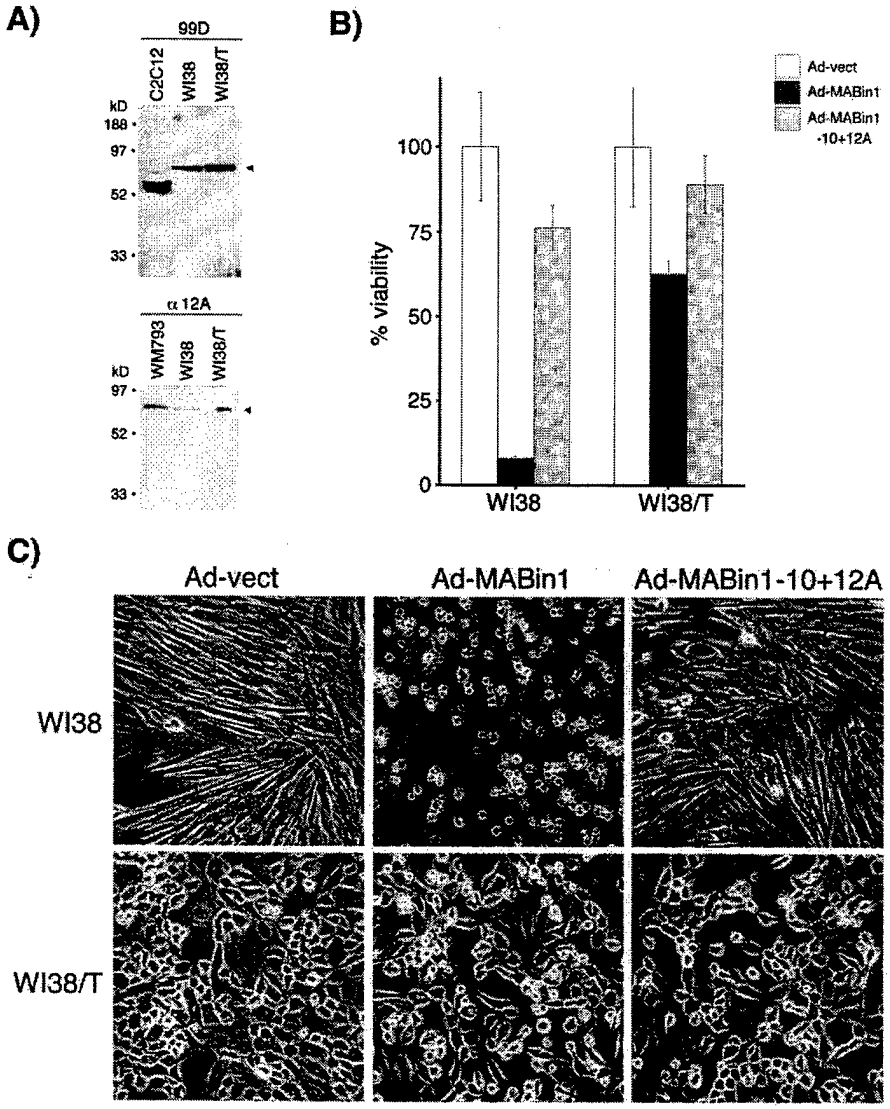


Figure 8 SV40 T antigen blocks cell death induction by Bin1. (a) Western analysis demonstrates missplicing of exon 12A in Bin1 in WI-38 diploid fibroblasts. Previous results from RT–PCR experiments demonstrated the presence of exon 12A in Bin1 messages expressed in WI-38 cells (Wechsler-Reya *et al*., 1997b). Top panel: extracts prepared from C2C12 myoblasts (positive control), WI-38 cells, and WI-38 cells transformed by SV40 T antigen (WI-38/T cells; also known as VA13 cells) were analysed with anti-Bin1 mAb 99D (Wechsler-Reya *et al*., 1997a). The arrow indicates a slower mobility band in WI-38 and WI-38/T consistent with the presence of aberrantly spliced isoform. Bottom panel: extracts prepared from the human melanoma cell line WM793 (positive control), WI-38 cells, and WI-38/T cells were analysed with anti-12A mAb (Ge *et al*., 1999). The arrow indicates a polypeptide including exon 12-derived residues in WI-38 and WI-38/T that has identical mobility to the aberrant Bin1-10+12A splice isoform expressed in human melanoma (Ge *et al*., 1999). (b) WI-38 cells are susceptible to Bin1 PCD, which is suppressed by SV40 T antigen. Cells were infected with 100 m.o.i. adenoviral vector indicated plus 100 m.o.i. Ad-cre and viable cells were determined by trypan blue exclusion 48 h later. (c) Cytopathic effect of Bin1 in WI-38 cells and its inhibition by SV40 T antigen. Cells were processed as above and photographed 48 h after viral infection

cytotoxic T cell granule protein granzyme B, or a variety of cytotoxic drugs, nor did other kinds of serine protease inhibitors affect death by c-Myc. Thus, AEBSF was a relatively specific inhibitor of a feature of the death process(es) induced by c-Myc. We reasoned that if there was any overlap in the mechanisms used by Bin1 and c-Myc, then AEBSF might affect the death process activated by Bin1. To test this notion, HepG2 cells were infected with Ad-LacZ or Ad-Bin1, or treated with staurosporine, and then left untreated or treated with AEBSF or its inactive analog AEBSA (4-(2-aminoethyl)benzenesulfonamide). Flow cytometery was used to monitor the appearance of sub-G1 phase DNA as a measurement of PCD. In contrast to ZVAD.fmk, AEBSF suppressed Bin1-induced DNA degradation to the background levels seen in cells infected with Ad-LacZ (see Figure 9). This suppression reflected inhibition of a serine protease(s), rather than some nonspecific effect, because the inactive compound AEBSA was ineffective. Consistent with previous findings that AEBSF is not a general inhibitor of apoptosis (Kagaya *et al*., 1997),

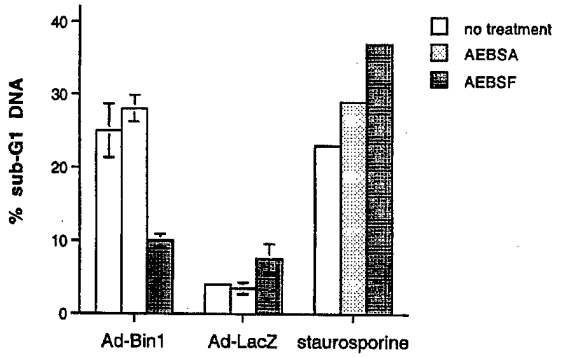


Figure 9 Implication of a serine protease(s) in programmed cell death by Bin1. HepG2 cells were infected with 100 m.o.i. Ad-Bin1 or Ad-LacZ or treated with 0.5 μM staurosporine and left untreated or treated additionally with 0.4 mM AEBSF or its inactive analog AEBSA. Cells were processed for flow cytometry 24 h later. The graph presents the relative proportion of the cell population exhibiting sub-G1 phase DNA. The mean and standard error of three trials is shown

under the same conditions where AEBSF suppressed the action of Bin1 it did not suppress DNA degradation induced by staurosporine (the effects of which were actually accentuated by AEBSF treatment). Thus, AEBSF specifically inhibited DNA degradation by Bin1. We concluded Bin1 acted in part through activation of a serine protease, similar to c-Myc, in support of the conclusion that Bin1 and c-Myc shared certain PCD mechanisms.

Discussion

Bin1 activated a PCD process in malignant cells

This study defines a function for the BAR family adaptor protein Bin1 in a caspase-independent PCD process that limits neoplastic transformation. Bin1 function is complex and regulated by alternate splicing. Splice isoforms that interact with c-Myc have tumor suppressor properties and are missing or inactivated in breast carcinoma, metastatic prostate cancer, and malignant melanoma (Ge *et al.*, 1999, 2000a,b; Sakamuro *et al.*, 1996). The results of this study suggest that the antineoplastic effects of these Bin1 isoforms in malignant cells are a consequence of their ability to activate PCD. Caspase-independent death processes where cytosolic features predominate without accompanying classical nuclear phenotypes have been termed type II apoptosis. The mechanisms underlying this type of PCD are poorly understood. Two other cancer suppression genes, Pml and CD47, have been reported to activate suicide programs with similar features. Pml is a nuclear dot-associated protein that is disrupted in acute promyelocytic leukemia. Expression of wild-type Pml in malignant cells engages a death program with features similar to that engaged by Bin1 (Quignon *et al.*, 1998). CD47 is a receptor for the antiangiogenic extracellular matrix protein thrombospondin. CD47 ligation engages a death program on chronic lymphocytic leukemia cells (Mateo *et al.*, 1999). It is unclear whether these programs share any common aspect. Nevertheless, such caspase-independent processes may be important because they are evolutionarily ancient: 'classic' apoptotic regulators such as CED-4, Bak, and Bax elicit death in yeast, with vacuolation phenotypes similar to those seen in metazoan cells, despite the fact that yeast lacks caspases (Ink *et al.*, 1997; James *et al.*, 1997; Jurgensmeier *et al.*, 1997; Xiang *et al.*, 1996). Such programs may be integrated yet distinct from apoptosis, which apparently evolved later in metazoan cells.

Independence from mitochondrial processes and caspases

We found that PCD by Bin1 was not subject to suppression by Bcl-2 or to inhibition of the Fas pathway and that cellular demise was not associated with mitochondrial alterations. These observations were internally consistent given that the death process was caspase-independent. However, they were unexpected given that Bin1 interacts with c-Myc, which activates classical apoptosis by eliciting cytochrome c release and subsequent caspase activation (Juin *et al.*, 1999).

Two important issues impact consideration of this apparent conundrum. First, it seems likely that c-Myc acts in a complex manner to induce death. Cytochrome c release has been shown to be an important component of the process activated by c-Myc, but this event is apparently insufficient (Juin *et al.*, 1999). In addition, as noted above, careful investigations have shown that neither Bcl-2 nor caspase inhibitors block the death commitment decision induced by c-Myc, but rather the phenotype and kinetics of the execution step (McCarthy *et al.*, 1997). Interestingly, caspase-independent suicides that occur retain the cytosolic but not the nuclear features of classical apoptosis (McCarthy *et al.*, 1997), not unlike the death phenotype induced by Bin1. It is important to draw a distinction between the mechanisms which affect death kinetics and those which affect death commitment decisions, because in slow growing tumors where c-Myc is overexpressed (such as many carcinomas) the effects of Bcl-2 or other antiapoptotic signals may be insufficient to provide adequate escape from the death penalty that is associated with c-Myc overexpression. If this is the case, there would be a selection for loss of caspase-independent processes that impact execution and that are separate from the mitochondrial and death receptor axes.

A second issue is the possibility that caspase-independent steps are required for apoptotic outcomes by c-Myc or other proapoptotic stimuli. A precedent for this concern is raised by studies of the tumor suppressor Pml, which activates caspase-independent death in tumor cells but which is also necessary for apoptosis induced by a wide variety of death stimuli (Quignon *et al.*, 1998; Wang *et al.*, 1998). It is interesting that the death processes used by c-Myc or Bin1 are each susceptible to inhibition at some level by AEBSF, a serine protease inhibitor which does not generally affect apoptosis (Kagaya *et al.*, 1997). Serine death proteases and caspases appear to act in various cells in different but integrated hierarchies (Kagaya *et al.*, 1997; Wright *et al.*, 1997), so the fact caspases are differentially activated by c-Myc and Bin1 supports the notion of more than one death signal emerging from c-Myc. Results from our laboratory using dominant

inhibitory and antisense approaches support the possibility that Bin1 may be necessary for apoptosis by c-Myc (unpublished observations). However, due to the unusual caspase-independent nature of the Bin1 death phenotype in malignant cells, we are addressing this question further using cells targeted for Bin1 gene deletion. In summary, despite the key role of apoptotic escape in malignant development, it is notable that caspases and other apoptosome components may be inactivated in cancer cells less frequently than caspase-independent functions such as Pml or Bin1 (Ge *et al.*, 1999, 2000a,b; Mu *et al.*, 1994; Sakamuro *et al.*, 1996; Zhang *et al.*, 2000). Our findings suggest that Bin1 participates in some caspase-independent process which can influence cell death commitment in malignant cells.

Susceptibility to inhibition by SV40 T antigen

We found that the death process activated by Bin1 was susceptible to suppression by SV40 large T antigen. The ability of this tumor virus protein to block the death process supported the notion that its inactivation is relevant to cancer development. This result was also consistent with earlier findings that transformation of rodent fibroblasts by T antigen is not subject to suppression by Bin1 (Sakamuro *et al.*, 1996). This observation does not conflict with the finding that Bin1 acts independently of p53 and Rb, because there is evidence that T antigen acts beyond inactivating p53 and Rb in immortalization and human cell transformation (Conzen and Cole, 1995; Hahn *et al.*, 1999; Powell *et al.*, 1999). For example, T antigen cooperates with activated Ras and the telomerase catalytic subunit TERT to cause malignant transformation of human cells, and one can not complement the effects of T antigen by coexpression of the human papilloma virus E6 and E7 proteins (Hahn *et al.*, 1999), which inactivate p53 and Rb, respectively. In this context, it is interesting to note that before the discovery of TERT the rare successes achieved in immortalizing human cells were achieved usually with T antigen or SV40 DNA. Interestingly, Bin1 is inactivated by missplicing in diploid WI-38 cells in the same manner as in malignant melanoma (Ge *et al.*, 1999) and this event makes them susceptible to killing by Bin1, unlike IMR diploid fibroblasts. Thus, Bin1 activation may be relevant to WI-38 biology, perhaps affecting some process also targeted by T antigen.

Bin1 function

Bin1 has two functions that are varied by alternate splicing. The most significant alterations in Bin1 occur in brain. Brain isoforms localize to clathrin-coated vesicles and promote synaptic vesicle endocytosis, like amphiphysin, by recruiting enzymes that modify lipids and alter membrane structure (Wigge and McMahon, 1998). In contrast, the ubiquitous Bin1 isoforms lack sequences needed for targeting to clathrin-coated vesicles (Ramjaun and McPherson, 1998) and instead exhibit nuclear localization (Elliott *et al.*, 1999; Wechsler-Reya *et al.*, 1997a, 1998). The c-Myc-interacting isoform analysed in this study did not affect endocytosis under conditions where it activated cell death. Preliminary examination of Bin1-10, a closely related c-Myc-interacting isoform, suggest it has similar properties (unpublished observations). In contrast, isoforms that include brain exon 12A did not induce death, consistent with previous results (Ge *et al.*, 1999). Thus, we believe that the death induction function of Bin1 is a unique feature of isoforms that can localize to the nucleus and interact with c-Myc. Bin1 isoforms form heterodimers in the brain (Wigge *et al.*, 1997). Therefore, it is tempting to speculate that the endocytosis connection in neurons reflects a specialized link in those cells between survival and the achievement of a synaptically active state, which would be associated with neurotransmitter release and hence endocytotic membrane trafficking.

From a functional standpoint, although ubiquitous Bin1 isoforms are not amphiphysin-like, the high conservation between amphiphysin and the ubiquitous Bin1 isoforms nonetheless suggest a functional connection to membrane dynamics at some level (perhaps related to internal vesicle dynamics). The BAR domain comprises the major part of this conservation. This domain is crucial for death activity but what it does is currently obscure. A membrane connection is intriguing in light of emerging interest in possible connections between autophagy and apoptosis (Thompson and Vander Heiden, 1999). However, we have not observed any death inhibitory effects of 3-methyladenine, a classical inhibitor of autophagy (unpublished observations). Nevertheless, there is certainly a precedent for a nucleocytosolic adaptor protein that has dual functions in transcriptional regulation and membrane dynamics. CtBP/BARS is an adaptor protein initially identified through its ability to interact with and inhibit the tumorigenic activity of adenovirus E1A (Schaeper *et al.*, 1995). CtBP/BARS binds to cell cycle and transcriptional regulatory complexes (Meloni *et al.*, 1999; Sewalt *et al.*, 1999; Sollerbrant *et al.*, 1996) but it also controls membrane vesiculation in the Golgi complex (Weigert *et al.*, 1999). Parallels between CtBP and Bin1 are intriguing given their connections to E1A and c-Myc, which are biologically distant cousins. A complex nature for Bin1, like that exhibited by CtBP/BARS, would not be out of step with the complex and still largely obscure nature of c-Myc, which has a highly integrated cell regulatory function.

We did not detect any changes in the expression of several target genes linked to apoptosis by c-Myc, including ornithine decarboxylase, CDC25A, or Fas ligand (Galaktionov *et al.*, 1996; Hueber *et al.*, 1997; Packham and Cleveland, 1994), but no target gene identified to date has been assigned an unambiguous role in apoptosis (Dang, 1999; Evan and Littlewood, 1998). In transient assays, Bin1 can suppress c-Myc transactivation and repress transcription when tethered to DNA (Elliott *et al.*, 1999). It is unclear whether these activities are physiologically germane; if they are epiphenomenon of Myc-Bin1 interaction transient assays, then Bin1 may have a signaling role that is unaffiliated with transactivation. The latter possibility can be entertained since not all biological actions of c-Myc can be ascribed strictly to gene regulation (Gusse *et al.*, 1989; Lemaitre *et al.*, 1995; Li *et al.*, 1994; Prendergast and Cole, 1989; Yang *et al.*, 1991) and because Bin1 more resembles a signaling protein than a transcription factor. Based on existing data, we have suggested that PCD by c-Myc involves distinct

'priming' and 'triggering' steps, the former of which is associated with gene regulation but the latter of which is not (Prendergast, 1999). Further investigations are required to unravel the physiological relationship between Bin1 and the proapoptotic and transcriptional properties of c-Myc.

Bin1, cell death, and cancer

Cancer is characterized by dysfunctional adhesion and cell survival signaling. Our findings suggest that loss of Bin1 may eliminate one mechanism which can limit the consequences of inappropriate activation of c-Myc or other oncogenes. Suicide mechanisms are progressively eliminated during neoplastic progression (Williams, 1991), and in invasive breast cancers and metastatic prostate cancers where Bin1 losses occur frequently (Ge *et al.*, 2000a,b) there is strong evidence that loss of cell suicide capacity corresponds with malignant conversion (Kyprianou *et al.*, 1990, 1991; McDonnell *et al.*, 1992). However, malignant conversion is associated generally with altered adhesive capabilities that facilitate invasion and metastasis. Resistance to anoikis (adhesion deprival-induced cell suicide) is likely to be a crucial feature of the pathophysiology of epithelial malignancy. Interestingly, cell death by many oncogenes including c-Myc is suppressed by integrin signaling (Crouch *et al.*, 1996), and Bin1 has been reported to interact with certain α-integrins (Wixler *et al.*, 1999). The interaction between Abl kinase and Bin1 in cells (Kadlec and Pendergast, 1997) may be consistent with an integrin connection, given evidence that Abl can become activated at focal adhesions where integrins are localized (Lewis *et al.*, 1996; Taagepera *et al.*, 1998). Therefore, further investigation of the links between Bin1-dependent cell death and integrin signaling may provide insights into the significance of Bin1 losses in cancer as well as to mechanisms that govern cell death induction by c-Myc and other oncogenes.

Materials and methods

Recombinant adenoviruses

Constitutive and inducible adenoviral vectors were developed by standard methods. The constitutive virus Ad-Bin1 contained Bin1 cDNA driven by a cytomegalovirus (CMV) enhancer-promoter in place of the E1 region of the virus. Briefly, the full-length Bin1 cDNA 99fE, that includes exons 1–11 and 13–16 but not brain-specific exons 12A–12D (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997b), was subcloned into pAdCMVlink-1. 293 cells were cotransfected with this derivative plus ClaI-digested dl7001 adenovirus DNA to obtain the recombinant virus by homologous recombination as described (Davis and Wilson, 1996). Similar viral vector constructions used Bin1-10+12A isoform, an aberrant tumor-specific and loss-of-function isoform (Ge *et al.*, 1999), or Bin1ΔBAR-C, a loss-of-function deletion mutant lacking a.a. 126–207 (Elliott *et al.*, 1999b). Plaque-purified clones were identified by Southern and Western analysis and the DNA sequence of the insert was determined to verify wild-type status. A variation of up to several hours was noted in the kinetics of death induction by different preparations of Ad-Bin1. The inducible virus Ad-MABin1 was constructed similarly in a cre-loxP adenoviral system kindly provided by FL Graham (Anton and Graham, 1995). Briefly, the luciferase cDNA in the vector pMA19 (Anton and Graham, 1995) was replaced with the 99fE cDNA. The resulting plasmid was cotransfected into 293 cells as above to obtain the recombinant virus by homologous recombination. In this virus, the Bin1 cDNA is conditionally expressed under the control of the cytomegalovirus (CMV) promoter, from which it is separated by an loxP-flanked stuffer sequence. Bin1 expression is suppressed in the unrearranged virus because the stuffer region includes stop codons in all three reading frames. The analogous Ad-MA19 virus, which expresses luciferase, was a gift of FL Graham. In cells expressing P1 bacteriophage Cre site-specific recombinase, the intervening stuffer region is removed by Cre-mediated excision, leading to expression of the transgene. Ad-vect is a control adenovirus kindly provided by JM Wilson which is similar in structure to the others except that it contains no transgene (Davis and Wilson, 1996).

Cell culture

HepG2, SAOS-2, and 293 cell lines were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 50 U/ml penicillin and streptomycin (Fisher). For adenovirus infections, HepG2 or SAOS-2 cells were plated at 5×10^5 cells per 6 cm culture dish and allowed to recover overnight. Virus was added to the cells at the indicated m.o.i. in a volume of 1 ml of DMEM supplemented with 2% fetal bovine serum and allowed to adhere for 2–3 h at 37°C. Cells were then washed and fed with DMEM 10% FBS. Where appropriate, the indicated concentrations of the broad spectrum caspase inhibitor ZVAD.fmk (Enzyme Systems Products) was added to cells at the time of infection and maintained until harvesting. Thapsigarin and staurosporine (Calbiochem) were used as chemical inducers of apoptosis at concentrations of 2 and 0.5 μM, respectively, and as positive controls produced similar results. For cell viability determinations, cells were harvested 48 h postinfection by trypsinization, washed in PBS, stained with trypan blue and counted using a hemacytometer. Where indicated, the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Sigma) or its inactive analog 4-(2-aminoethyl)benzenesulfonamide (AEBSA) (Aldrich), which substitutes an amino group for the crucial fluoride moiety in AEBSF. Both inhibitors were added to cells at infection and maintained at a final concentration of 0.4 mM, a concentration which is nontoxic but sufficient to suppress c-Myc-mediated apoptosis (Kagaya *et al.*, 1997). For electron microscopy, HepG2 cells were infected with Ad-LacZ or Ad-Bin1 (m.o.i.=100) or treated with 0.5 μM staurosporine and where indicated treated with 100 μM ZVAD.fmk. Adherent and floating cells were harvested 36 h after adenoviral infection or 24–30 h after staurosporine treatment and processed for osmium tetroxide staining and electron microscopy using standard methods. Baculovirus experiments were performed in the insect cell line Sf9 using full-length recombinant viruses expressing Bin1, Bcl-2 or no insert, essentially as described (Alnemri *et al.*, 1992; Elliott *et al.*, 1999b). Viable cell counts were determined at the indicated times after virus infection by the trypan blue exclusion method.

Western analysis

Cell lysates was prepared and analysed by standard protocols (Harlow and Lane, 1988). Briefly, lysates were fractionated by SDS–PAGE, gels were electrophoretically transferred to ECL membrane (Amersham) or Immobilon-P (Millipore). Blots were blocked in 3% skim milk and probed with anti-Bin1 monoclonal antibodies 99D, 99I or α12A, or anti-Bcl-2 antibody #124 (DAKO). 99D and 99I recognize epitopes in the c-Myc binding domain encoded by exon 13 whereas α12A recognizes an epitope encoded by brain-specific exon 12A (Wechsler-Reya *et al.*, 1997a; Ge *et al.*, 1999). Antibodies

were diluted 1:50 in PBS with 2.5% skim milk and 0.1% Tween-20 and incubated with the membrane 12 h at 4°C. Blots were washed and incubated 1 h in the same buffer with secondary goat anti-mouse antibodies conjugated to horseradish peroxidase (BMB) and developed using a chemiluminescence kit using the protocol suggested by the vendor (Pierce).

Immunofluorescence

Cells were seeded on coverslips and the next day infected with recombinant adenoviruses or treated with drugs. After the periods indicated cells were washed twice with PBS, stained 5 min with 5 μg/ml Hoechst 33323 (Sigma) dissolved in PBS, and analysed immediately by immunofluorescence. For endocytosis assays, cells seeded on coverslips were infected with recombinant adenoviruses and 20 h later processed for fluid-phase uptake or receptor-mediated endocytosis as described (Barbieri *et al*., 1998; Benmerah *et al*., 1998). Briefly, cells were incubated 30 min in serum-free DMEM and then treated with 100 nM fluorescein-conjugated transferrin (Sigma) (receptor-mediated) or 2 mg/ml horseradish peroxidase (Sigma) in 0.2% BSA (fluid-phase). After incubation for 15 min, cells were cooled to 4°C, washed 2× with ice-cold PBS, and fixed in 3.7% paraformaldehyde. Cells were mounted for analysis by confocal fluorescence microscopy or processed with horseradish peroxidase substrate before microscopic analysis.

Flow cytometry

Cells were harvested by trypsinization and fixed in 1% paraformaldehyde and then 80% ethanol. TUNEL assay was performed by incubating samples 1 h at 37°C in TdT reaction buffer (Boehringer Mannheim) and then staining 30 min at room temperature with fluoresceinated streptavidin (DuPont). Cells were then incubated 30 min at room temperature with propidium iodide. Alternatively, to analyse only DNA degradation, cells were fixed in 70% ethanol and permeabilized in PBS/0.2% Tween-20 followed by propidium iodide staining as described (Sakamuro *et al*., 1997). Flow cytometry was performed on a EPIC/XL cell analyser (Coulter).

Nucleosomal DNA assay

Genomic DNA was prepared by a modified Hirt method (Debbas and White, 1993; Hirt, 1967) or by the following method, as indicated. Cells were gently resuspended in 250 μl TBE buffer (90 mM Tris-borate/2 mM EDTA) containing 0.4% NP40 and 5 μg RNAse A and incubated 30 min at 37°C. Proteinase K and SDS were added to 100 μg/ml and 1% (v/v), respectively, and samples were incubated overnight at 55°C. Genomic DNA was extracted with phenol-chloroform, precipitated with ethanol, and fractionated by agarose gel electrophoresis.

Caspase activity assay

Lysates prepared from equivalent numbers of cells were used to assess caspase-3-like activities, employing the Quantizyme Assay System as recommended by the vendor (Biomol Research Laboratories). Briefly, duplicate plates were harvested 48 h post-infection, one for cell counts and the other for cell lysates. 5×10^7 cells/ml were extracted in the cell lysis buffer provided by the vendor and 10 μl of extract was used in reactions to monitor cleavage of the substrate Ac-DEVD-pNA. Reactions were examined at various times after addition of the substrate by spectroscopy at 405 nM to monitor release of the product. Data were analysed with DeltasoftII Software (Biometallics Inc.).

Acknowledgments

For providing cDNA clones we thank D Andrews, D Beach, S Frisch, S Nagata, R Press and D Quelle. Support from the Wistar Flow Cytometry Core Facility and the Wistar Cancer Core Grant CA10815 is acknowledged. We thank T Halazonetis, A Oliff, E Routhier and A Muller for critical comments. This work was funded by grants from the US Army Breast Cancer Research Program (DAMD17-96-1-6324) and the American Cancer Society (CN-160). K Elliott was supported by an NIH training grant. K Ge was the recipient of a fellowship award from the Adler Foundation. GC Prendergast is a Pew Scholar in the Biomedical Sciences.

References

Alnemri ES, Robertson NM, Fernandes TF, Croce CM and Litwack G. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 7295–7299.

Amarante-Mendes GP, Finucane DM, Martin SJ, Cotter TG, Salvesen GS and Green DR. (1998). *Cell Death Differ.*, **5**, 298–306.

Anton M and Graham FL. (1995). *J. Virol.*, **69**, 4600–4606.

Ashkenazi A and Dixit VM. (1998). *Science*, **281**, 1305–1308.

Barbieri MA, Kohn AD, Roth RA and Stahl PD. (1998). *J Biol. Chem.*, **273**, 19367–19370.

Benmerah A, Lamaze C, Begue B, Schmid SL, Dautry-Varsat A and Cerf-Bensussan N. (1998). *J. Cell Biol.*, **140**, 1055–1062.

Bowen C, Spiegel S and Gelmann EP. (1998). *Cancer Res.*, **58**, 3275–3281.

Butler MH, David C, Ochoa G-C, Freyberg Z, Daniell L, Grabs D, Cremona O and De Camilli P. (1997). *J. Cell Biol.*, **137**, 1355–1367.

Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA and Gruss P. (1998). *Cell*, **94**, 727–737.

Chao DT and Korsmeyer SJ. (1998). *Ann. Rev. Immunol.*, **16**, 395–419.

Cole MD. (1986). *Ann. Rev. Genet.*, **20**, 361–384.

Conzen SD and Cole CN. (1995). *Oncogene*, **11**, 2295–2302.

Crouch DH, Fincham VJ and Frame MC. (1996). *Oncogene*, **12**, 2689–2696.

Dang CV. (1999). *Mol. Cell. Biol.*, **19**, 1–11.

David C, McPherson PS, Mundigl O and de Camilli P. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 331–335.

Davis AR and Wilson JM. (1996). *Current Protocols in Human Genetics*. John Wiley & Sons, Inc.: New York, pp 12.4.1–18.

Day ML, Foster RG, Day KC, Zhao X, Humphrey P, Swanson P, Postigo AA, Zhang SH and Dean DC. (1997). *J. Biol. Chem.*, **272**, 8125–8128.

Debbas M and White E. (1993). *Genes Dev.*, **7**, 546–554.

Ding HF, McGill G, Rowan S, Schmaltz C, Shimamura A and Fisher DE. (1998). *J. Biol. Chem.*, **273**, 28378–28383.

Elliott K, Sakamuro D, Basu A, Du W, Wunner W, Staller P, Gaubatz S, Zhang H, Prochownik E, Eilers M and Prendergast GC. (1999). *Oncogene*, **18**, 3564–3573.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S. (1998). *Nature*, **391**, 45–50.

Evan G and Littlewood T. (1998). *Science*, **281**, 1317–1322.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell*, **69**, 119–128.

Fearnhead HO, McCurrach ME, O'Neill J, Zhang K, Lowe SW and Lazebnik YA. (1997). *Genes Dev.*, **11**, 1266–1276.

Galaktionov K, Chen X and Beach D. (1996). *Nature*, **382**, 511–517.
Garte SJ. (1993). *Crit. Rev. Oncog.*, **4**, 435–449.
Ge K, DuHadaway J, Du W, Herlyn M, Rodeck U and Prendergast GC. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 9689–9694.
Ge K, DuHadaway J, Sakamuro D, Wechsler-Reya R, Reynolds C and Prendergast GC. (2000a). *Int. J. Cancer*, **85**, 376–383.
Ge K, Minhas F, DuHadaway J, Mao N-C, Wilson D, Sakamuro D, Buccafusca R, Nelson P, Malkowicz SB, Tomaszewski JT and Prendergast GC. (2000b). *Int. J. Cancer*, **86**, 155–161.
Green DR and Reed JC. (1998). *Science*, **281**, 1309–1312.
Gusse M, Ghysdael J, Evan G, Soussi T and Mechali M. (1989). *Mol. Cell. Biol.*, **9**, 5395–5403.
Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA. (1999). *Nature*, **400**, 464–468.
Harlow E and Lane D. (1998). *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
Hirt B. (1967). *J. Mol. Biol.*, **26**, 365–369.
Hueber AO, Zornig M, Lyon D, Suda T, Nagata S and Evan GI. (1997). *Science*, **278**, 1305–1309.
Ink B, Zornig M, Baum B, Hajibagheri N, James C, Chittenden T and Evan G. (1997). *Mol. Cell. Biol.*, **17**, 2468–2474.
James C, Gschmeissner S, Fraser A and Evan GI. (1997). *Curr. Biol.*, **7**, 246–252.
Jenkins RB, Qian J, Lieber MM and Bostwick DG. (1997). *Cancer Res.*, **57**, 524–531.
Juin P, Hueber AO, Littlewood T and Evan G. (1999). *Genes Dev.*, **13**, 1367–1381.
Jurgensmeier JM, Krajewski S, Armstrong RC, Wilson GM, Oltersdorf T, Fritz LC, Reed JC and Ottilie S. (1997). *Mol. Cell. Biol.*, **18**, 325–339.
Kadlec L and Pendergast A-M. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 12390–12395.
Kagaya S, Kitanaka C, Noguchi K, Mochizuki T, Sugiyama A, Asai A, Yasuhara N, Eguchi Y, Tsujimoto Y and Kuchino Y. (1997). *Mol. Cell. Biol.*, **17**, 6736–6745.
Kangas A, Nicholson DW and Hottla E. (1998). *Oncogene*, **16**, 387–398.
Kyprianou N, English HF, Davidson NE and Isaacs JT. (1991). *Cancer Res.*, **51**, 162–166.
Kyprianou N, English HF and Isaacs JT. (1990). *Cancer Res.*, **50**, 3748–3753.
Lazebnik YA, Cole S, Cooke CA, Nelson WG and Earnshaw WC. (1993). *J. Cell Biol.*, **123**, 7–22.
Lazebnik YA, Takahashi A, Moir RD, Goldman RD, Poirier GG, Kaufmann SH and Earnshaw WC. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 9042–9046.
Lemaitre JM, Bocquet S, Buckle R and Mechali M. (1995). *Mol. Cell Biol.*, **15**, 5054–5062.
Lewis JM, Baskaran R, Taagepera S, Schwartz MA and Wang JY. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 15174–15179.
Li L, Nerlov C, Prendergast G, MacGregor D and Ziff EB. (1994). *EMBO J.*, **13**, 4070–4079.
Liu X, Zou H, Slaughter C and Wang X. (1997). *Cell*, **89**, 175–184.
Mao NC, Steingrimsson EJD, Ruiz J, Wasserman W, Copeland NG, Jenkins NA and Prendergast GC. (1999). *Genomics*, **56**, 51–58.
Mateo V, Lagneaux L, Bron D, Biron G, Armant M, Delespesse G and Sarfati M. (1999). *Nat. Med.*, **5**, 1277–1284.
McCarthy NJ, Whyte MKB, Gilberg CS and Evan GI. (1997). *J. Cell Biol.*, **136**, 215–227.
McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM and Campbell ML. (1992). *Cancer Res.*, **52**, 6940–6944.
Meloni AR, Smith EJ and Nevins JR. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 9574–9579.
Mu ZM, Chin KV, Liu JH, Lozano G and Chang KS. (1994). *Mol. Cell. Biol.*, **14**, 6858–6867.
Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME and Dixit VM. (1996). *Cell*, **85**, 817–827.
Packham G and Cleveland JL. (1994). *Mol. Cell. Biol.*, **14**, 5741–5747.
Powell AJ, Darmon AJ, Gonos ES, Lam EW, Peden KW and Jat PS. (1999). *Oncogene*, **18**, 7343–7350.
Prendergast GC. (1999). *Oncogene*, **18**, 2966–2986.
Prendergast GC and Cole MD. (1989). *Mol. Cell. Biol.*, **9**, 124–134.
Quignon F, De Bels F, Koken M, Feunteun J, Ameisen JC and de The H. (1998). *Nat. Genet.*, **20**, 259–265.
Ramjaun AR and McPherson PS. (1998). *J. Neurochem.*, **70**, 2369–2376.
Ramjaun AR, Micheva KD, Bouchelet I and McPherson PS. (1997). *J. Biol. Chem.*, **272**, 16700–16706.
Rao L, Perez D and White E. (1996). *J. Cell Biol.*, **135**, 1441–1455.
Reed JC, Jurgensmeier JM and Matsuyama S. (1998). *Biochim. Biophys. Acta*, **1366**, 127–137.
Sakamuro D and Prendergast GC. (1999). *Oncogene*, **18**, 2942–2953.
Sakamuro D, Elliott K, Wechsler-Reya R and Prendergast GC. (1996). *Nature Genet.*, **14**, 69–77.
Sakamuro D, Eviner V, Elliott K, Showe L, White E and Prendergast GC. (1995). *Oncogene*, **11**, 2411–2418.
Sakamuro D, Sabbatini P, White E and Prendergast GC. (1997). *Oncogene*, **15**, 887–898.
Schaeper U, Boyd JM, Verma S, Uhlmann E, Subramanian T and Chinnadurai G. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 10467–10471.
Sewalt RG, Gunster MJ, van der Vlag J, Satijn DP and Otte AP. (1999). *Mol. Cell. Biol.*, **19**, 777–787.
Sherr CJ. (1998). *Genes Dev.*, **12**, 2984–2991.
Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW and Lowe SW. (1999). *Science*, **284**, 156–159.
Sollerbrant K, Chinnadurai G and Svensson C. (1996). *Nuc. Acids Res.*, **24**, 2578–2584.
Suzuki A, Tsutomi Y, Akahane K, Araki T and Miura M. (1998). *Oncogene*, **17**, 931–939.
Taagepera S, McDonald D, Loeb JE, Whitaker LL, McElroy AK, Wang JY and Hope TJ. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 7457–7462.
Thompson CB and Vander Heiden MG. (1999). *Nat. Cell Biol.*, **1**, E209–E216.
Trudel M, Lanoix J, Barisoni L, Blouin MJ, Desforges M, L'Italien C and D'Agati V. (1997). *J. Exp. Med.*, **186**, 1873–1884.
Tsuneoka M and Mekada E. (2000). *Oncogene*, **19**, 115–123.
Tsutsui K, Maeda Y, Tsutsui K, Seki S and Tokunaga A. (1997). *Biochem. Biophys. Res. Comm.*, **236**, 178–183.
Wang ZG, Ruggero D, Ronchetti S, Zhong S, Gaboli M, Rivi R and Pandolfi PP. (1998). *Nat. Genet.*, **20**, 266–272.
Wechsler-Reya R, Elliott K, Herlyn M and Prendergast GC. (1997a). *Cancer Res.*, **57**, 3258–3263.
Wechsler-Reya R, Elliott K and Prendergast GC. (1998). *Mol. Cell. Biol.*, **18**, 566–575.
Wechsler-Reya R, Sakamuro D, Zhang J, Duhadaway J and Prendergast GC. (1997b). *J. Biol. Chem.*, **272**, 31453–31458.

Weigert R, Silletta MG, Spanol S, Turacchio G, Cericola C, Colanzi A, Senatore S, Mancini R, Polishchuk EV, Salmona M, Facchiano F, Burger KN, Mironov A, Luini A and Corda D. (1999). *Nature,* **402,** 429–433.

Wigge P, Kohler K, Vallis Y, Doyle CA, Owen D, Hunt SP and McMahon HT. (1997). *Mol. Biol. Cell,* **8,** 2003–2015.

Wigge P and McMahon HT. (1998). *Trends Neurosci.,* **21,** 339–344.

Williams GT. (1991). *Cell,* **65,** 1097–1098.

Wixler V, Laplantine E, Geerts D, Sonnenberg A, Petersohn D, Eckes B, Paulsson M and Aumailley M. (1999). *FEBS Lett.,* **445,** 351–355.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW and Mak TW. (1998). *Genes Dev.,* **12,** 806–819.

Wright SC, Schellenberger U, Wang H, Kinder DH, Talhouk JW and Larrick JW. (1997). *J. Exp. Med.,* **186,** 1107–1117.

Xiang J, Chao D and Korsmeyer S. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 14559–14563.

Yang B-S, Geddes TJ, Pogulis RJ, de Crombrugghe B and Freytag SO. (1991). *Mol. Cell. Biol.,* **11,** 2291–2295.

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM and Mak TW. (1998). *Cell,* **94,** 739–750.

Zhang P, Chin W, Chow LTC, Chan ASK, Yim APC, Leung S-F, Mok TSK, Chang K-S, Johnson PJ and Chan JYH. (2000). *Int. J. Cancer,* **85,** 599–605.

Genomics 67, 210–220 (2000)
doi:10.1006/geno.2000.6216, available online at http://www.idealibrary.com on IDEAL®

Bin2, a Functionally Nonredundant Member of the BAR Adaptor Gene Family

Kai Ge*,[1] and George C. Prendergast*,†,[2]

*The Wistar Institute, Philadelphia, Pennsylvania 19104; and †Glenolden Laboratory, DuPont Pharmaceuticals Company, Glenolden, Pennsylvania 19036

Received January 31, 2000; accepted April 5, 2000

BAR family proteins are a unique class of adaptor proteins characterized by a common N-terminal fold of undetermined function termed the BAR domain. This set of adaptors, which includes the mammalian proteins amphiphysin and Bin1 and the yeast proteins Rvs167p and Rvs161p, has been implicated in diverse cellular processes, including synaptic vesicle endocytosis, actin regulation, differentiation, cell survival, and tumorigenesis. Here we report the identification and characterization of Bin2, a novel protein that contains a BAR domain but that is otherwise structurally dissimilar to other members of the BAR adaptor family. The Bin2 gene is located at chromosome 4q22.1 and is expressed predominantly in hematopoietic cells. Bin2 is upregulated during differentiation of granulocytes, suggesting that it functions in that lineage. Bin2 formed a stable complex in cells with Bin1, but not with amphiphysin, in a BAR domain-dependent manner. This finding indicates that BAR domains have specific preferences for interaction. However, Bin2 did not influence endocytosis in the same manner as brain-specific splice isoforms of Bin1, nor did it exhibit the tumor suppressor properties inherent to ubiquitous splice isoforms of Bin1. Thus, Bin2 appears to encode a nonredundant function in the BAR adaptor gene family.

INTRODUCTION

BAR proteins are a family of adaptor proteins implicated in a diverse set of cellular processes, including cell growth control, cell survival, differentiation, endocytosis, and actin organization. This family is characterized by a common N-terminal domain termed the BAR domain and includes the mammalian Bin1 and amphiphysin proteins and the yeast Rvs167 and Rvs161 proteins. Amphiphysin, the first member of the BAR family to be identified, was named for its biochemical properties rather than for its cellular function (Lichte *et al.,* 1992). Subsequent studies showed that amphiphysin is a cytosolic neuronal protein and functions in synaptic vesicle endocytosis (Wigge and McMahon, 1998). Amphiphysin also acts as a paraneoplastic autoimmune antigen in rare cancers of the breast, lung, and other tissues that are associated clinically with certain nervous system disorders (Antoine *et al.,* 1999; Dropcho, 1996; Folli *et al.,* 1993). Amphiphysin can be detected in breast and certain other nonneuronal tissues (Floyd *et al.,* 1999), but it is expressed predominantly in the central nervous system.

The yeast proteins Rvs167p and Rvs161p were identified in a screen for mutations that caused reduced viability upon nutrient starvation (Bauer *et al.,* 1993; Crouzet *et al.,* 1991). Yeast lacking these proteins can proliferate but cannot properly exit the cell cycle and sporulate when starved, suggesting roles in cell growth regulation. Rvs proteins are cytosolic and have been implicated in actin regulation and endocytosis (Balguerie *et al.,* 1999; Bauer *et al.,* 1993; Breton and Aigle, 1998; Colwill *et al.,* 1999; Lila and Drubin, 1997; Sivadon *et al.,* 1995). Rvs161p has a unique function in cell fusion during mating that can be separated from its role in endocytosis (Brizzio *et al.,* 1998). Rvs167p may have a unique role in linking actin regulation to the cell cycle, insofar as it is an important substrate for Pho85p (Lee *et al.,* 1998), a cell cycle kinase recently defined as the yeast homolog of the mammalian enzyme cdk5 (Huang *et al.,* 1999; Nishizawa *et al.,* 1999).

The mammalian Bin1 gene (bridging integrator-1) encodes a set of BAR proteins with different functions that are generated by tissue-specific splicing. Bin1 was identified initially through its ability to interact with and inhibit the oncogenic properties of the c-Myc oncoprotein (Sakamuro *et al.,* 1996). Subsequent analysis identified two ubiquitous splice isoforms and several tissue-specific splice isoforms expressed predominantly in skeletal muscle and brain (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997; Wechsler-Reya *et al.,* 1997b, 1998). Brain-specific splice isoforms are

Sequence data from this article have been deposited with the GenBank Data Library under Accession No. AF146531.

[1] Current address: The Rockefeller University, New York, NY 10012.

[2] To whom correspondence should be addressed. Telephone: (610) 237-7847. Fax: (610) 237-7937. E-mail: george.c.prendergast@dupontpharma.com.

0888-7543/00 $35.00

most closely related to amphiphysin and have been termed alternately amphiphysin II (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997; Wigge *et al.,* 1997; Wigge and McMahon, 1998). However, outside the brain it is clear that Bin1 has unique functions that are not amphiphysin-like. First, ubiquitous isoforms found outside the brain are localized in the nucleus as well as the cytosol, whereas brain-specific isoforms are apparently strictly cytosolic. Second, only brain-specific isoforms include sequences required for interaction with clathrin and elements of the endocytotic machinery (Ramjaun and McPherson, 1998). Although amphiphysin is commonly referred to as the ortholog of Rvs167, Bin1 may fulfill this role, since outside the brain it is closer in size and structure as well as ubiquitous rather than tissue restricted in expression. Nuclear isoforms that associate with c-Myc can suppress c-Myc-dependent gene activation and have been implicated in the control of cell proliferation, differentiation, and programmed cell death (Elliott *et al.,* 1999; Mao *et al.,* 1999; Prendergast, 1999; Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1998). Moreover, there is extensive evidence that c-Myc-interacting isoforms have tumor suppressor properties that are frequently missing or inactivated in human cancers, including malignant melanoma, invasive breast cancer, and metastatic prostate cancer (Ge *et al.,* 1999, 2000a, b). In contrast, amphiphysin and brain-specific splice isoforms of Bin1 lack tumor-suppressor activity. Significantly, one mechanism by which Bin1 is functionally inactivated in human cancer is by missplicing of a neuronal exon (Ge *et al.,* 1999). Thus, it is clear that while BAR adaptor proteins share a common domain they have divergent physiological functions.

In this study, we report the characterization of a novel mammalian BAR adaptor protein termed Bin2 (bridging integrator-2). The expression pattern of Bin2 is reminiscent of amphiphysin in that it appears to be largely tissue restricted, except that in the case of Bin2 it is found predominantly in hematopoietic cells rather than in the central nervous system. Interestingly, Bin2 lacks sequences found in Bin1 or amphiphysin that mediate interaction with c-Myc or with clathrin and the endocytotic machinery. Bin2 also lacks a C-terminal SH3 domain found in the other mammalian BAR proteins, instead including a unique long C-terminal extension. Consistent with these structural differences, Bin2 lacked endocytotic or antiproliferative properties displayed by amphiphysin and Bin1. Thus, Bin2 encodes a novel and nonredundant function in the BAR adaptor gene family.

MATERIALS AND METHODS

Cloning and plasmid vectors. A TBLASTN search of the expressed sequence tag (EST) database with the amino acid sequences encompassing the C-terminal of the BAR domain of Bin1 identified a germinal B cell cDNA (GenBank Accession No. AA452680) that encoded a polypeptide related to but nonidentical with amphiphysin or Bin1. Using this EST cDNA as a probe, a full-length cDNA was obtained from a human leukocyte 5′-Stretch Plus cDNA library (HL5019t; Clontech). The complete DNA sequence of this clone was determined by standard methods (GenBank Accession No. AF146531). Human genomic Bin2 BAC clones, used for chromosome mapping experiments, were isolated by Genome Systems, Inc., using the Bin2 cDNA fragment. An epitope-tagged Bin2 cDNA was engineered by the replacement of the initiator methionine with the influenza virus hemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 (Niman *et al.,* 1983). For *in vivo* expression Bin2 and HA-Bin2 were subcloned into the mammalian vector pcDNA3.1/neo (Invitrogen). A similar vector for expression of full-length Bin1 has been described (Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1998). Full-length cDNA for Bin1−10+12ABCD [also known as amphiphysin IIa (Ramjaun *et al.,* 1997)] or human amphiphysin was also subcloned into pcDNA3.1/neo for expression. BAR deletion mutants of Bin1 that lack the amino acid residues indicated were constructed as described (Elliott *et al.,* 1999) and subcloned for expression into pcDNA3.1/neo.

Fluorescence in situ hybridization (FISH) chromosome analysis. FISH was performed using a Bin2 genomic BAC clone labeled by nick-translation with digoxigenin–dUTP and metaphase chromosomes isolated from PHA-stimulated normal peripheral blood lymphocytes. A biotin-labeled probe specific for the centromere of chromosome 4 was also included in the hybridization. Slides were developed using fluoresceinated anti-digoxigenin antibodies and Texas red avidin. Two experiments were done and a total of 80 metaphase cells were analyzed with 73 exhibiting specific labeling. Map location was determined on chromosome 4 by measuring the physical distance on 10 labeled chromosomes, computed to be 27% of the distance from the centromere to the telomere of chromosome arm 4q, an area that corresponds to band 4q22.1.

Blot analyses. Genomic DNA was isolated and analyzed by Southern analysis by standard methods (Sambrook *et al.,* 1989) using full-length Bin2 cDNA as a hybridization probe. Total cytoplasmic RNA was prepared from adherent cell lines as described (Prendergast and Cole, 1989). Total RNA from suspension cell lines was isolated using a commercial kit employing TriZol reagent (Life Technologies). Fifteen micrograms of RNA per lane was fractionated on formaldehyde gels and analyzed essentially as described (Prendergast and Cole, 1989), using Bin2 cDNA as a hybridization probe.

Protein–protein interaction. Bin2 or HA-Bin2 was cotranslated *in vitro* with Bin1 or amphiphysin proteins using a commercial reticulocyte extract kit (Promega). Complex formation was assessed by immunoprecipitation with anti-HA antibody 12CA5 (BMB), anti-amphiphysin (Transduction Laboratory), or anti-Bin1 antibody 99D (Wechsler-Reya *et al.,* 1997a), followed by SDS–PAGE and fluorography. For *in vivo* experiments, 293T cells were transfected with vectors for Bin1, HA-Bin2, or no insert using LipofectAmine 2000 following the vendor's protocol (Life Technologies). Forty-eight to sixty h after transfection, cells were collected and lysed in ice-cold NP-40 lysis buffer [150 mM NaCl, 0.5% NP-40, 50 mM Tris–Cl (pH 8.0), 1 mM PMSF, 1 μg/ml leupeptin, 0.4 U/ml aprotinin, 10 μg/ml leupeptin]. Cell lysates were immunoprecipitated with Bin1 mAb 99D or anti-HA mAb 12CA5 and immunoblotted with 99D or the polyclonal anti-HA antibody sc-805 (Santa Cruz Biotechnology).

Tissue culture. Human tumor cell lines, 293T cells (ATCC CRL-1573), and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies) and 50 U/ml penicillin and streptomycin (Fisher). The human myeloid leukemia cell line HL60 was maintained in RPMI 1640 supplemented with 15% FCS (Life Technologies) and 50 U/ml penicillin and streptomycin (Fisher).

Endocytosis assay. COS cells were plated on coverslips and transfected 24 h later with expression vectors for Bin2 or the Bin1 brain-specific splice isoform Bin1−10+12ABCD [also known as amphiphysin IIa (Ramjaun *et al.,* 1997)]. Bin1−10+12ABCD has been shown to inhibit transferrin uptake in COS cells (Wigge *et al.,* 1997). A modified calcium phosphate protocol was used for transfection

A

```
MAEGKAGGAAGLFAKQVQKKFSRAQEKVLQKLGKAVETKDERFEQSASNFYQQQAEGHKLYKDLKNFLSAVKVMHESSKRVSET
LQEIYSSEWDGHEELKAIVWNNDLLWEDYEEKLADQAVRTMEIYVAQFSEIKERIAKRGRKLVDYDSARHHLEAVQNAKKDEAK
TAKAEEEFNKAQTVFEDLNQELLEELPILYNSRIGCYVTIFQNISNLRDVFYREMSKLNHNLYEVMSKLEKQHSNKVFVVKGLS
SSSRRSLVISPPVRTATVSSPLTSPTSPSTLSLKSESESVSATEDLAPDAAQGEDNSEIKELLEEEEIEKEGSEASSSEEDEPL
PACNGPAQAQPSPTTERAKSQEEVLPSSTTPSPGGALSPSGQPSSSATEVVLRTRTASEGSEQPKKRASIQRTSAPPSRPPPPR
ATASPRPSSGNIPSSPTASGGGSPTSPRASLGTGTASPRTSLEVSPNPEPPEKPVRTPEAKENENIHNQNPEELCTSPTLMTSQ
VASEPGEAKKMEDKEKDNKLISADSSEGQDQLQVSMVPENNNLTAPEPQEEVSTSENPQL
```

B

```
Bin2      1   MAE-GKAGGAAGLFAKQVQKKFSRAQEKVLQKLGKAVETKDERFEQSASNFYQQQAEGHK    59
              MAE G  G  AG  A  VQKK +RAQEKVLQKLGKA ETKDE+FEQ   NF +Q +EG +
Bin1      1   MAEMGSKGVTAGKIASNVQKKLTRAQEKVLQKLGKADETKDEQFEQCVQNFNKQLTEGTR    60
              MA         G+FAK VQK+  RAQEKVLQKLGKA ETKDE FE    NF  Q AEG +
Amphi     1   MADIK-----TGIFAKNVQKRLNRAQEKVLQKLGKADETKDEQFEEYVQNFKRQEAEGTR    55

Bin2     60   LYKDLKNFLSAVKVMHESSKRVSETLQEIYSSEWDGHEELKAIVWNNDLLWEDYEEKLAD   119
              L KDL+ +L++VK MHE+SK+++E LQE+Y  +W G +E   I  NNDLLW DY +KL D
Bin1     61   LQKDLRTYLASVKAMHEASKKLNECLQEVYEPDWPGRDEANKIAENNDLLWMDYHQKLVD   120
              LQ++L  +L+A+K M+E+S ++ E L E+Y  +W G E+   +    D+LWED+  KL D
Amphi    56   LQRELG-YLAAIKGMQEASMKLTESLHEVYEPDWYGREDVKMVGEDCDVLWEDFHQKLVD   115

Bin2    120   QAVRTMEIYVAQFSEIKERIAKRGRKLVDYDSARHHLEAVQNA-KKDEAKTAKAEEEFNK   178
              QA+ TM+ Y+ QF +IK RIAKRGRKLVDYDSARHH E++Q A KKDEAK AKAEEE  K
Bin1    121   QALLTMDTYLGQFPDIKSRIAKRGRKLVDYDSARHHYESLQTAKKKDEAKIAKAEEELIK   180
               ++ T + Y+ QF +IK RIAKR RKLVDYDSARHHLE+LQ++ +KDE++ +KAEEEF K
Amphi   116   GSLLTLDTYLGQFPDIKNRIAKRSRKLVDYDSARHHLEALQSSKRKDESRISKAEEEFQK   176

Bin2    179   AQTVFEDLNQELLEELPILYNSRIGCYVTIFQNISNLRDVFYREMSKLNHNLYEVMSKLE   238
              AQ VFE++N +L EELP L+NSR+G YV  FQ+I+ L + F++EMSKLN NL +V+  LE
Bin1    181   AQKVFEEMNVDLQEELPSLWNSRVGFYVNTFQSIAGLEENFHKEMSKLNQNLNDVLVGLE   240
              AQ VFE+ N +L EELP L+ SR+G YV  F N+S L   F++E + L H LYEVM+KL
Amphi   177   AQKVFEEFNVDLQEELPSLWSRRVGFYVNTFKNVSSLEAKFHKEIAVLCHKLYEVMTKLG   237

Bin2    239   KQHSNKVFVVKG
              KQH +  F VK
Bin1    241   KQHGSNTFTVKA
             +QH  K F + G
Amphi   238  DQHADKAFTIQG
```

FIG. 1. Structure of human Bin2 and its relationship to the BAR family of adaptor proteins. (**A**) Conceptual translation of full-length human Bin2 cDNA isolated from a leukocyte library. (**B**) Alignment of the BAR domains of Bin2, Bin1, and amphiphysin. The BLAST2 algorithm was used for alignment; amphiphysin was aligned manually. Identical or similar residues (the latter marked by a plus sign) are indicated relative to the Bin2 sequence. (**C**) BAR family adaptor proteins. The cartoon compares the domain structure of Bin2 with known BAR proteins in mammals (Bin1 and amphiphysin) and the yeast *Saccharomyces cerevisiae* (Rvs167p and Rvs161p). Different splice isoforms of Bin1 are noted; exon numbers are from Wechsler-Reya *et al.* (1997b).

(Chen and Okayama, 1987). Thirty h after transfection, cells were incubated with FITC-conjugated transferrin (Molecular Probes), and transferrin receptor-mediated endocytosis was monitored by indirect immunofluorescence as described (Benmarah *et al.*, 1999). Cells expressing HA-Bin2 or Bin1−10+12ABCD were identified with monoclonal antibodies anti-HA 12CA5 or anti-Bin1 99D, respectively (Niman *et al.*, 1983; Wechsler-Reya *et al.*, 1997a). A Texas red-conjugated horse anti-mouse antibody (Vector) was used to visualize the primary antibody.

RESULTS

Bin2 is a mammalian BAR adaptor protein that lacks an SH3 domain. We observed previously that a polyclonal Bin1 antiserum crossreacted with non-Bin1 polypeptides in cells that do not express amphiphysin (Sakamuro *et al.*, 1996), suggesting that additional BAR family adaptor proteins existed. Subsequent epitope mapping of this antiserum defined a major epitope at the extreme C-terminus of the BAR domain (Wechsler-Reya *et al.*, 1997a). A TBLASTN search of the EST database with amino acid sequences derived from this region identified a partial germinal B cell cDNA (GenBank Accession No. AA452680) that encoded a polypeptide related to but nonidentical to amphiphysin and Bin1. This cDNA was used as a probe to isolate full-length cDNA clones from a human leukocyte phage library. DNA sequence analysis identified a long open reading frame of 564 aa rich in serine and glutamic acid that has a predicted molecular mass of 61709 Da (see Fig. 1A). Southern analysis using the cDNA probe confirmed the presence of Bin2 sequences in human genomic DNA (data not shown). Comparison of Bin1 to the polypeptide encoded by this cDNA, which was termed Bin2, confirmed the presence of a complete BAR motif that was 61% identical and 75% similar to Bin1 with boundaries of aa 1–249 in Bin2 and aa 1–251 in Bin1 (see Fig. 1B). Bin2 was identical to Bin1 within the region of the BAR domain that is most highly conserved in evolution (aa 138–155). Amphiphysin has a nonidentical residue in this region and was slightly less similar overall as computed by the BLAST algorithm. A second segment of the BAR domain that was

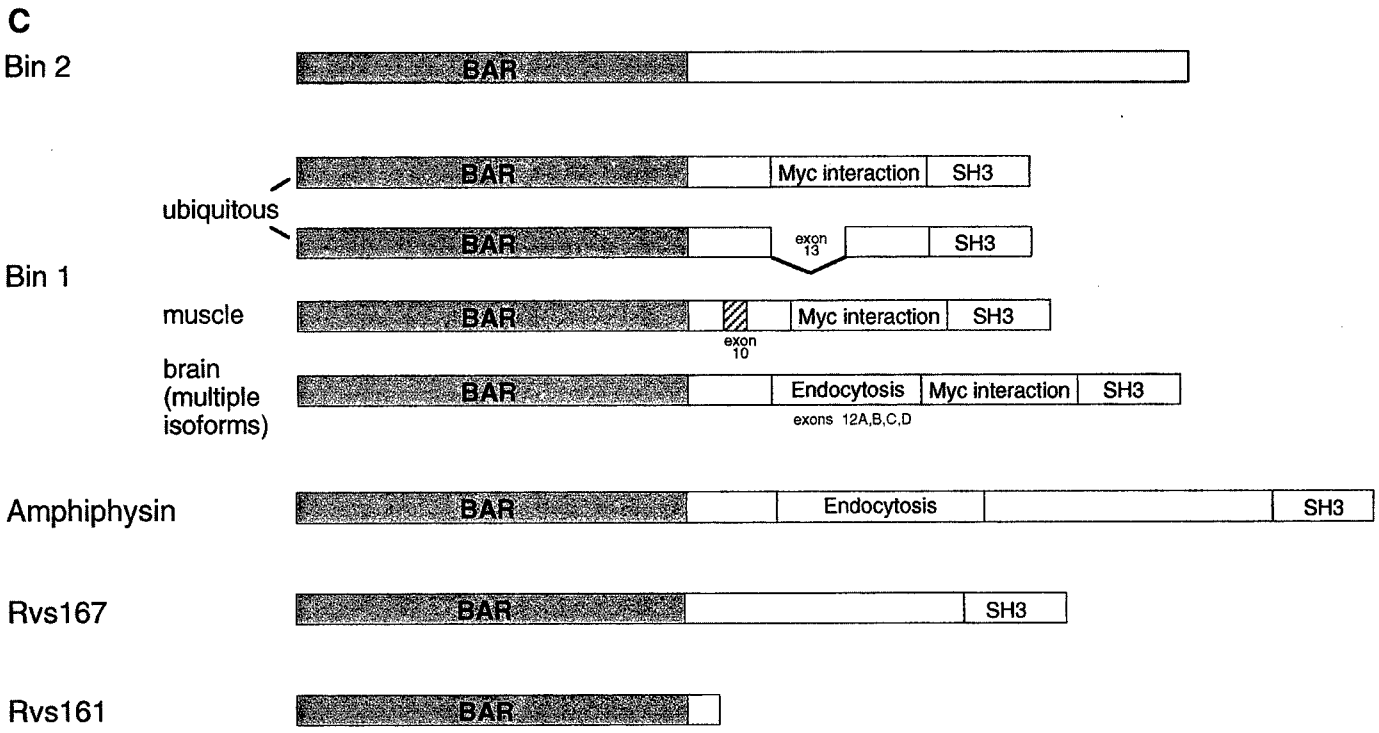


FIG. 1—*Continued*

highly conserved in these proteins was aa 23–45, which encompasses a region predicted to be helical in configuration (data not shown). The high degree of conservation between the N-terminal region of Bin2 and the BAR domains of Bin1 and amphiphysin identified Bin2 as a third member of the BAR family to be identified in mammalian cells.

Outside the BAR domain Bin2 lacked any similarity to other members of the BAR adaptor family (see Fig. 1C). The C-terminal regions in this family exhibit considerable structural diversity, especially in the case of Bin1, which is alternately spliced constitutively or in a tissue-specific manner. The C-terminal extension in Bin2 (aa 250–564) included acidic and serine/proline-rich segments but was otherwise unrelated to either BAR family adaptors or other known proteins. One possible exception was a distant relationship noted by database comparisons using the BLAST algorithm between the midsection of Bin2 (aa 179–336) and a central region of Daxx, a ubiquitous protein implicated in a variety of nuclear processes connected to transcription and programmed cell death (Chang *et al.,* 1998; Michaelson *et al.,* 1999; Torii *et al.,* 1999). The *E* value computed for the BLAST sequence alignment (which measures its relative significance) was higher than the analogous alignment between Bin2 and Rvs161 (9×10^{-3} versus 7.6×10^{-2}, respectively). Nevertheless, since the score was relatively low the significance of this relationship was uncertain. As noted above, Bin2 lacked a C-terminal SH3 domain that is found in all BAR adaptor proteins except Rvs161 (Fig. 1C). However, while Rvs161 lacks an SH3 it also lacks a C-terminal extension. Thus, Bin2 seems unlikely to be

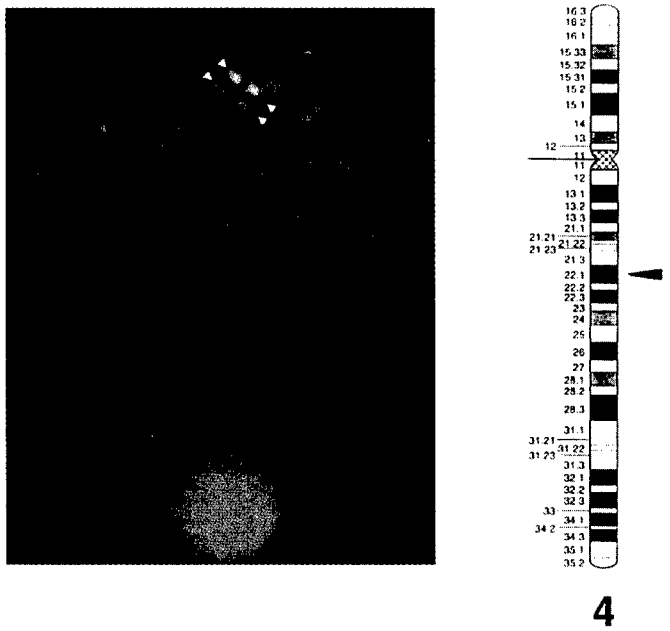


FIG. 2. The human Bin2 gene localizes to chromosome 4q22.1. Chromosomal location of Bin2 was mapped by FISH of metaphase chromosomes isolated from PHA-stimulated normal peripheral blood lymphocytes. Probes were a Bin2 genomic BAC clone labeled with digoxigenin–dUTP and a biotin-labeled DNA fragment that is specific for the centromere of chromosome 4. Slides were developed with fluoresceinated anti-digoxigenin antibodies and Texas red avidin. The white arrowheads indicate a representative pattern hybridization of 73 metaphase chromosome spreads analyzed, with red color identifying the chromosome 4 centromere and green color identifying the Bin2 locus on the long arm of the chromosome. The right side presents the map location of Bin2 as computed by determining the average physical distance between labeled loci on 10 separate chromosomes, an area that corresponds to band 4q22.1.

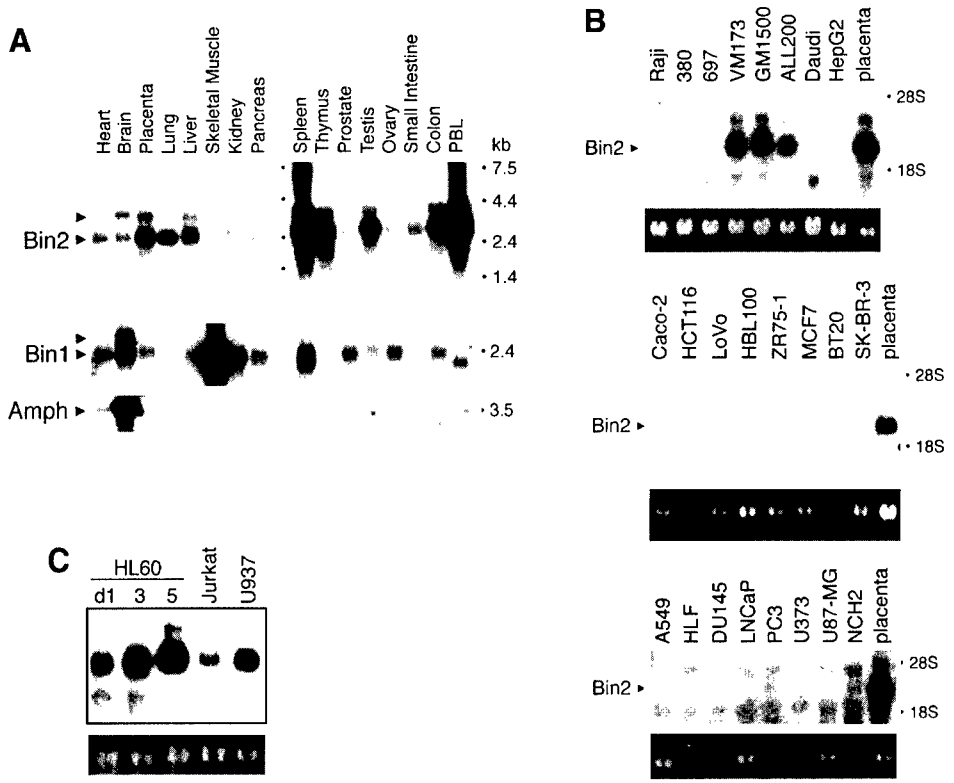


FIG. 3. Bin2 is expressed predominantly in hematopoietic cells and is induced during granulocytic differentiation. RNAs isolated from the tissues and cell lines indicated were subjected to Northern analysis using Bin2 cDNA as a hybridization probe. Ethidium bromide-stained gels are provided as RNA loading controls. (**A**) Human tissues. Blots were stripped and rehybridized sequentially to Bin1 and amphiphysin cDNA probes to contrast the patterns of expression of mammalian BAR proteins. An apparent splice isoform(s) of Bin2 is indicated by the upper arrowhead in the top blot. The several major splice isoforms of Bin1 seen specifically in brain (Wechsler-Reya *et al.,* 1997b) are indicated by the upper arrowhead in the middle blot. A ubiquitous splice isoform of Bin1 (second ubiquitous form shown in Fig. 1C) predominates in hematopoietic cells and can be seen in spleen, thymus, and peripheral blood lymphocytes (PBL). Amphiphysin expression is essentially confined to brain. (**B**) Human cell lines. Placenta and HepG2 hepatoma cells are positive and negative controls for expression, respectively. Top: lymphoid cell lines. Middle and bottom: adherent cell lines derived from breast, colon, lung, liver, prostate, glial cell, and astrocyte. (**C**) Bin2 is induced during granulocytic differentiation. HL60 promyelocytic leukemia cells were treated for the times indicated with DMSO to induce granulocytic differentiation, and RNA was isolated for Northern analysis with Bin2 cDNA. Jurkat is a T lymphoid cell line and U937 is a myeloid cell line to compare expression in other positive blood cell lines.

the mammalian ortholog of Rvs161 but instead a gene that arose later in evolution. Bin2 also lacked sequences required in Bin1 for interaction with c-Myc (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996) or in amphiphysin or brain-specific splice isoforms of Bin1 for interaction with clathrin and other elements of the endocytotic machinery (Ramjaun and McPherson, 1998; P. de Camilli, New Haven, CT, pers. comm., 10 Jan. 1999). Figure 1C summarizes the structural features of BAR proteins. Taken together, the structural differences between Bin2 and other members of the BAR adaptor family suggested that Bin2 encoded a nonredundant function. We concluded that Bin2 was a novel mammalian BAR protein likely to play a unique role in cells.

The human Bin2 gene is located on chromosome 4q22.1 and exhibits aberrant organization in hepatoma cells. The Bin2 cDNA was used to isolate three human genomic BAC clones by standard methods. Restriction analysis and Southern hybridization of these clones and comparison to genomic Southern hybridizations confirmed the presence of Bin2 sequences and ruled out the possibility that a pseudogene was cloned. One of the clones, F727, was used to perform FISH analysis of metaphase chromosomes isolated from normal peripheral blood lymphocytes. Specific hybridization signals were detected on the long arm of a group B chromosome consistent with chromosome 4 on the basis of size, morphology, and banding pattern. An experiment that included a second probe specific for the centromere sequences of chromosome 4 confirmed this interpretation. A total of 80 metaphase cells were analyzed, with 73 exhibiting specific labeling (see Fig. 2). Measurements of 10 specifically labeled chromosomes 4 demonstrated that the Bin2-specific hybridization signal was located at a position 27% of the distance from the centromere to the telomere of chromosome arm 4q, an area corresponding to band 4q22.1.

Bin2 is expressed predominantly in hematopoietic cells and is upregulated during monocytic differentiation. Northern analyses of human tissues and cell lines were performed to investigate the range of expression of Bin2 and to compare it with amphiphysin and Bin1 expression (see Fig. 3). The major message hybridized was ~2.6 kb, which was in reasonable

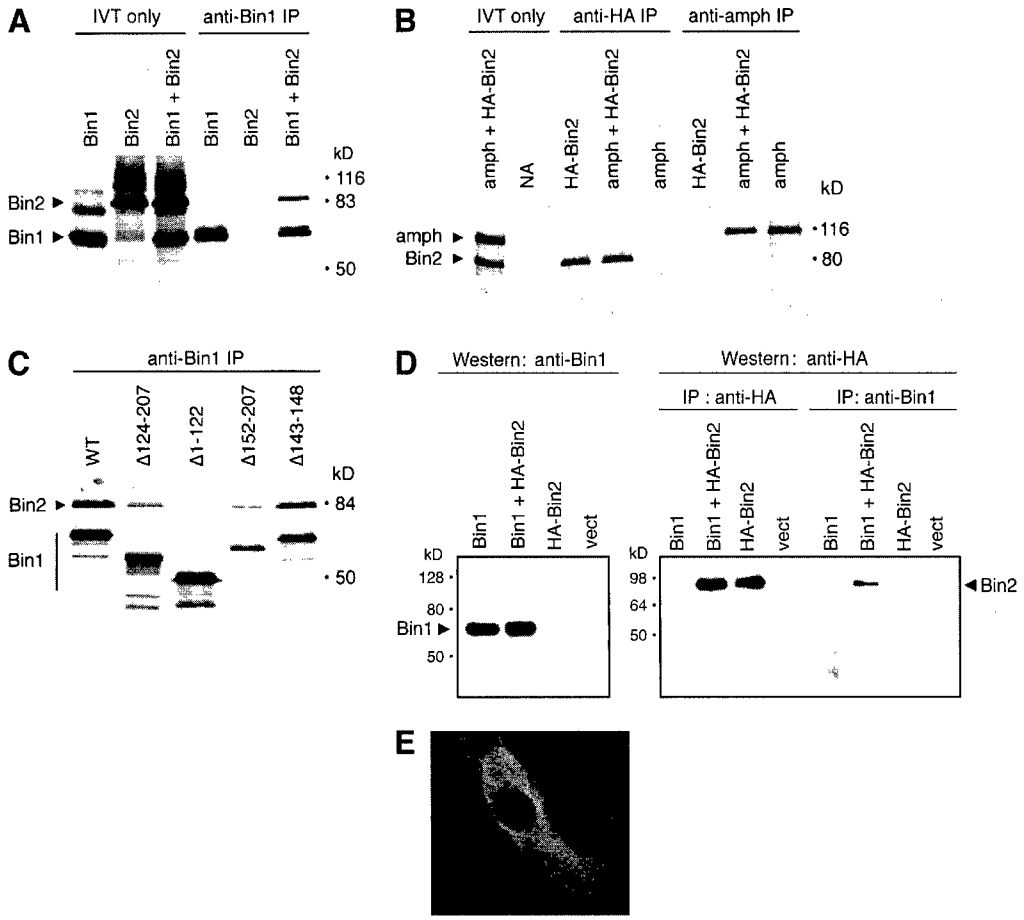


FIG. 4. Bin2 forms a stable BAR domain-dependent complex with Bin1 but not with amphiphysin. (**A**) Bin2 associates stably with Bin1. Bin2 and Bin1 cDNAs were subjected to *in vitro* translation with empty vector or with each other in the presence of [^{35}S]methionine. Two microliters of IVT products was fractionated directly on SDS–PAGE (IVT only); 10 μl of IVT products was subjected to immunoprecipitation with anti-Bin1 antibody 99D (Wechsler-Reya *et al.,* 1997a) before SDS–PAGE fractionation and fluorography. (**B**) Bin2 does not interact with amphiphysin. HA-Bin2 and amphiphysin cDNAs were subjected to *in vitro* translation and immunoprecipitation as above, using anti-HA 12CA5 or anti-amphiphysin. (**C**) N-terminal sequences in the Bin1 BAR domain are crucial for association with Bin2. A set of Bin1 deletion mutants lacking the amino acid residues indicated (Sakamuro *et al.,* 1996) was cotranslated with Bin2 and subjected to immunoprecipitation, SDS–PAGE, and fluorography as above. (**D**) Bin2 and Bin1 associate *in vivo.* COS cells were transfected with expression vectors for HA-Bin2 or Bin1 or with no insert. Cell extracts were prepared 2 days later and subjected to IP–Western analysis with anti-Bin1 and anti-HA antibodies. The left shows the results of straight Western analysis with anti-Bin1. The right shows Western analysis using anti-HA antibody of immunoprecipitates generated by either anti-HA or anti-Bin1. (**E**) Bin2 is cytosolic. COS cells were transfected as above with HA-Bin2 and processed for indirect immunofluorescence as described (Wechsler-Reya *et al.,* 1998), using anti-HA antibody to identify Bin2 and Texas red-conjugated anti-mouse IgG to visualize the antigen.

agreement with the ~2.2-kb cDNA characterized, but an additional message of ~3.5 kb was expressed at lower levels in several tissues (see Fig. 3A, top). The hybridization stringency in the experiment was high, suggesting that these messages were derived by alternate splicing, as seen in Bin1 (see Fig. 3A, middle), but a distinct isoform was not ruled out. Notably, steady-state RNA levels were at least two magnitudes higher in spleen and peripheral blood leukocytes than in other tissues and significantly higher in thymus, colon, and placenta than in other tissues. This pattern of expression suggested that Bin2 might be preferentially expressed in hematopoietic cells (colon and placenta are comparatively rich in lymphocytes and monocytes, respectively). The pattern of Bin2 expression contrasted with Bin1, which was ubiquitous, with highest expression in brain and muscle, and with amphiphysin, which was confined essentially to brain (see Fig. 3A, bottom).

Additional experiments supported the notion that Bin2 was expressed predominantly in blood cells and that its presence in most tissues was a consequence of its high expression in hematopoietic cells that are present in tissue isolates. First, Bin2 was undetectable by Northern analysis of 21 human cell lines derived from a variety of tissues, including breast, lung, prostate, brain, connective tissue (fibroblast), liver, and colon, despite detection of Bin2 in these tissues. In contrast, Bin2 message was strongly expressed in several human lymphoid and lymphoid cell lines, including GM1500, ALL200, BV173, and HL60 (see Fig. 3B). Second, "virtual" analyses performed by comparing

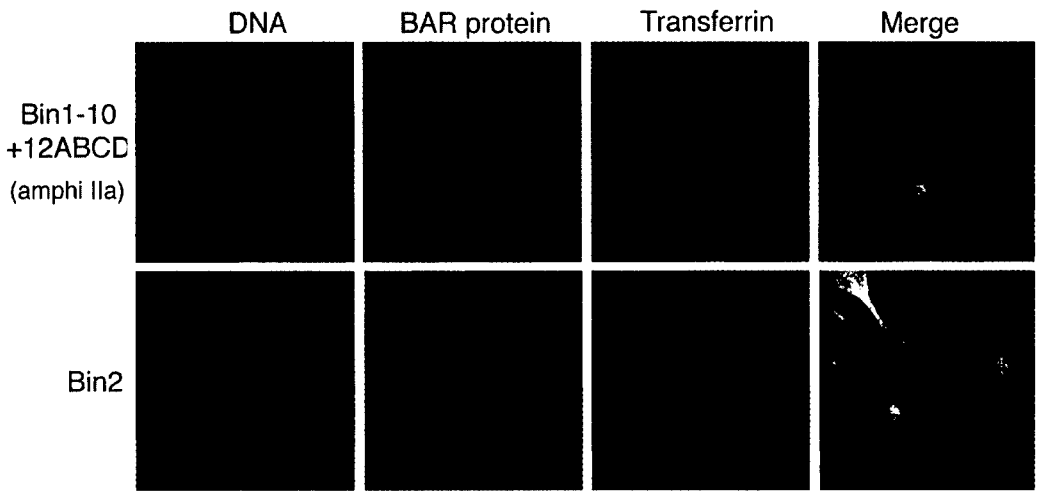


FIG. 5. Bin2 does not inhibit receptor-mediated endocytosis. Transferrin uptake as a measurement of receptor-mediated endocytosis was assayed essentially as described (Benmerah *et al.*, 1999). Briefly, COS cells were transfected with vectors for Bin1–10+12ABCD (amphiphysin IIa) or HA-Bin2 and incubated 24 h later with fluorescein-conjugated transferrin. All cells were stained with DAPI to visualize DNA. Bin1–10+12ABCD and Bin2 were detected with 99D (1:100 v/v hybridoma supernatant) or anti-HA 12CA5 (4 μg/ml), respectively, using a Texas red-conjugated secondary antibody (1:200 v/v) to visualize the proteins for immunofluorescence microscopy. Images were collected and processed using a confocal microscope apparatus.

Bin2 sequences to the EST database, which provides information about the tissue source for cDNA libraries (which enrich for tissue-specific messages), offered an additional line of confirmation that Bin2 is expressed mainly in blood cells (data not shown). The likelihood that the lack of expression in clonal cell lines was due to *in vitro* selection against antiproliferative activity was ruled out (see below). In summary, the results argued that Bin2 was expressed predominantly in blood cells.

Consistent with a functional role in blood lineages, we observed that Bin2 was induced during granulocytic differentiation of HL60 cells, a promyelocytic leukemia cell line. HL60 cells are induced to differentiate to granulocytes by treatment with dimethyl sulfoxide (DMSO). Northern analysis demonstrated increased steady-state levels of Bin2 RNA after 5 days of DMSO treatment relative to early times or to control Jurkat T cells or U937 myeloid cells (see Fig. 3C). The elevation of Bin2 during myeloid differentiation was reminiscent of a similar elevation of Bin1 that occurs during myoblast differentiation (Mao *et al.*, 1999; Wechsler-Reya *et al.*, 1998). We concluded that Bin2 was expressed predominantly in hematopoietic cells and that it was likely to function in granulocytes and other blood cells.

Bin2 is a cytosolic protein that associates with Bin1, but not amphiphysin, in a BAR-dependent manner. Rvs161 and Rvs167 form complexes in yeast, and amphiphysin and Bin1 form stable complexes in brain extracts (Navarro *et al.*, 1997; Wigge *et al.*, 1997). We therefore examined the ability of Bin2 to interact with Bin1 or amphiphysin *in vitro* and *in vivo.* Bin2 cDNA was subjected to *in vitro* translation (IVT) in the presence or absence of Bin1 or amphiphysin and complex formation was assessed by coimmunoprecipitation (IP) with anti-Bin1 antibody 99D (Wechsler-Reya *et al.*, 1997a) or an anti-amphiphysin antibody, followed by SDS–PAGE and fluorography. For some experiments, a derivative of Bin2 was constructed that included an N-terminal influenza HA epitope tag that is recognized by the monoclonal antibody 12CA5 (Niman *et al.*, 1983). Expression of this HA-tagged derivative, termed HA-Bin2, was confirmed by DNA sequencing and by *in vitro* translation (data not shown). Bin2 migrated with an apparent mobility of ~80 kDa, greater than the predicted molecular mass of ~61 kDa but reminiscent of the similar aberrant mobility displayed by Bin1 on Laemmli gels (Sakamuro *et al.*, 1996).

Interestingly, Bin2 formed a stable complex with Bin1 but not with amphiphysin. Interaction with Bin1 was consistent with evidence that BAR proteins form oligomers (Navarro *et al.*, 1997; Wigge *et al.*, 1997). The Bin2–Bin1 complex was stable under the conditions used and was approximately equimolar (see Fig. 4A). Notably, under the same conditions Bin2 and amphiphysin did not form stable complexes (see Fig. 4B). This result suggested either that these proteins could not form a complex or that their interaction was relatively unstable. Using a set of Bin1 deletion mutants (Elliott *et al.*, 1999), we mapped the determinants needed for interaction with Bin2. Deletion of the so-called unique central region (white boxes in Fig. 1C), the Myc-binding domain, or the SH3 domain had no effect on the efficiency of Bin2 co-IP (data not shown). In contrast, deletions within the BAR domain reduced or abolished interaction (see Fig. 4C). A major requirement for interaction mapped to the N-terminal region of Bin1 (aa 1–122), which includes one of the highly conserved segments of the BAR domain (see Fig. 1B). In contrast, deletion of sequences comprising the C-terminal half of the BAR domain (aa 124–207) reduced association only about twofold. A second deletion within this region (aa 152–207) produced a similar effect, consistent with some contribution of this region

to the efficiency of Bin2–Bin1 interaction. In contrast, deletion of a short segment within the most highly conserved part of the BAR domain (aa 143–148) did not affect interaction at all, supporting the notion that these sequences have a different function (Elliott *et al.,* 1999). In summary, the results indicated that N-terminal BAR sequences in Bin1 were crucial for specific complex formation with Bin1 and that conserved C-terminal BAR sequences had a lesser or different role.

To confirm that Bin1 and Bin2 could associate stably *in vivo* we conducted a similar set of IP experiments from extracts derived from COS cells transiently transfected with mammalian expression vectors. COS cells were transfected with Bin1 and HA-Bin2 vectors and then processed for IP–Western analysis by standard methods. HA-Bin2 was observed specifically in Bin1 immunoprecipitates (see Fig. 4D). Similar results were obtained by substituting in the experiment the brain-specific isoform Bin1−10+12ABCD, arguing that different splice isoforms of Bin1 that included identical BAR domains could associate with Bin2 similarly (data not shown). Bin1 splice isoforms vary in their subcellular localization, so we examined the localization of Bin2 in COS cells transfected with HA-Bin2 and processed for indirect immunofluorescence with anti-HA antibody. Bin2 was localized exclusively in the cytosol (see Fig. 4E). We concluded that Bin2 was a cytosolic protein that associated stably with Bin1 in cells in a BAR domain-dependent manner.

Bin2 does not influence receptor-mediated endocytosis. Brain-specific splice isoforms of Bin1 inhibit receptor-mediated endocytosis when overexpressed in COS cells (Wigge *et al.,* 1997). To determine whether Bin2 functions similarly, we compared its ability to inhibit transferrin uptake relative to the brain-specific isoform Bin1−10+12ABCD. Briefly, COS cells were transfected with HA-Bin2 or Bin1−10+12ABCD, incubated 24 h later with fluorescein-conjugated transferrin, and processed for indirect immunofluorescence as described (Benmarah *et al.,* 1999). Cells were stained with DAPI, to locate cell nuclei, and anti-HA or 99D, to locate cells expressing HA-Bin2 or Bin1−10+12ABCD, respectively.

We observed no effect of Bin2 overexpression on transferrin endocytosis in this model (see Fig. 5). Bin1−10+12ABCD markedly inhibited transferrin uptake, as indicated by greatly reduced fluorescein signal in transfected cells. In contrast, untransfected cells in the same microscope field were brightly stained, illustrating high background levels of transferrin endocytosis under these conditions. Bin2 lacked similar activity. Cells transfected with HA-Bin2 exhibited similar levels of fluorescein signal, relative to untransfected cells in the same microscope field. In addition, merged fields showed yellow signal, illustrating overlap of green and red signals for transferrin and Bin2 protein, respectively, confirming that Bin2 did not inhibit transferrin uptake (compare Merge, Fig. 5). We concluded that Bin2 does not function similarly to Bin1 in receptor-mediated endocytosis.

Bin2 lacks antiproliferative activity and does not affect the tumor-suppressor properties of Bin1. Bin1 has tumor-suppressor properties in malignant cells (Elliott *et al.,* 1999; Ge *et al.,* 1999, 2000a, b; Sakamuro *et al.,* 1996), so we investigated whether Bin2 may have similar effects and/or whether it could influence the growth-inhibitory activity of Bin1. For these experiments we employed several malignant cell lines that were susceptible to Bin1 suppression and that lack Bin2 expression (thereby offering a useful null background), including HepG2 hepatoma, MCF-7 breast, A549 lung, and DU145 and PC3 prostate cells. Cells were transfected with the same expression vector used above, which carries a neomycin-resistance cassette, and stably transformed cells were selected in growth medium containing G418. Unlike the case with Bin1 (Sakamuro *et al.,* 1996), we observed little if any effect on colony formation efficiency relative to empty vector. HepG2 exhibited an approximately twofold suppression relative to A549 and other cell lines tested, which showed no difference in colony formation efficiency (see Fig. 6A). To determine whether the apparent suppression of HepG2 reflected growth inhibition, we cloned and expanded a set of G418-resistant HepG2 colonies derived from Bin2 transfection and determined whether the cells stably expressed Bin2. Northern analysis indicated robust expression of Bin2 in all independent cell lines examined (see Fig. 6B). Moreover, we did not see any detrimental effect on cell proliferation (data not shown). Thus, Bin2 expression was compatible with *in vitro* proliferation of all the malignant cells tested, including HepG2. To investigate the possible effects of Bin2 on growth suppression by Bin1, we performed a similar set of colony formation experiments in HepG2 cells, except that cells were cotransfected with untagged empty vector or Bin2 vector and a neomycin-resistance gene-tagged Bin1 vector. Bin1 suppressed colony formation, consistent with previous observations (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996), and cotransfection of Bin2 did not markedly influence this effect (see Fig. 6C). We concluded that Bin2 lacked the tumor-suppressor features inherent to Bin1.

DISCUSSION

This study reports the molecular cloning and characterization of Bin2, the third mammalian BAR protein to be identified. The structure, expression patterns, and preliminary functional analysis of Bin2 each point to a nonredundant function for this protein in cells. Bin2 lacked features seen in the mammalian relatives Bin1 and amphiphysin, which have been implicated in certain signal transduction events related, respectively, to c-Myc and c-Abl in the nucleus (Elliott *et al.,* 1999; Kadlec and Pendergast, 1997; Sakamuro *et al.,* 1996) and to endocytosis in the cytosol (Butler *et*

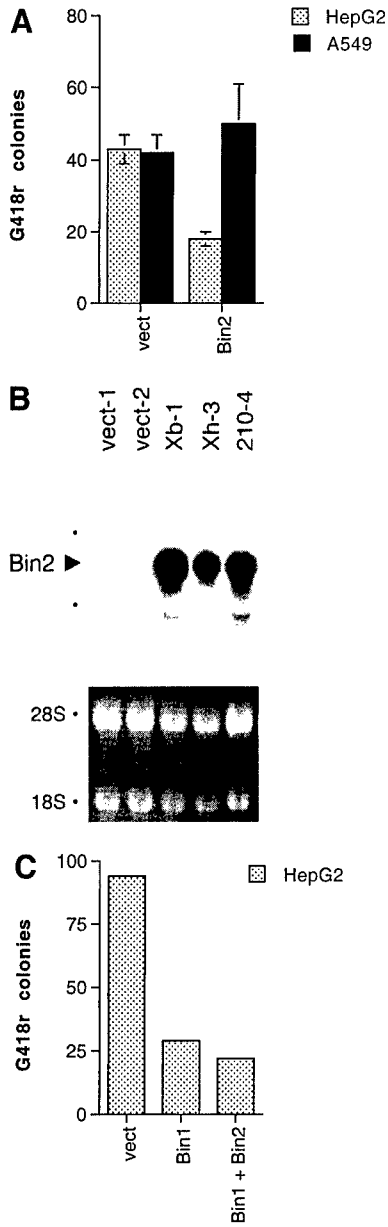


FIG. 6. Bin2 lacks antiproliferative activity and does not affect the tumor suppressor properties of Bin1. (**A**) Colony formation assay. HepG2 hepatoma or A549 lung carcinoma cells were transfected with expression vectors containing a neomycin-resistance gene cassette and Bin2 cDNA or no insert. Stable transformants were selected by culturing cells in G418. Colonies were scored ~3 weeks later by methanol fixation and crystal violet staining and were counted. The results depict the means and standard errors from three trials. (**B**) Northern analysis of HepG2 cell lines derived from transfection with Bin2 or empty vectors. Stably transformed colonies were ring-cloned and expanded into cell lines. RNA was isolated from cells and subjected to Northern analysis using Bin2 cDNA as a hybridization probe. The bottom shows the ethidium bromide-stained gel before transfer as a loading control. (**C**) Bin2 does not affect the tumor suppressor activity of Bin1. Colony formation assay was performed as above. Briefly, HepG2 cells were cotransfected with the expression vectors indicated, and stably transformed colonies were selected in G418.

al., 1997; Ramjaun and McPherson, 1998; Ramjaun *et al.,* 1997; Wigge *et al.,* 1997; Wigge and McMahon, 1998). Bin2 is the first mammalian BAR protein found that lacks an SH3 domain. However, although this is also a feature of the yeast Rvs161 protein, the presence of a C-terminal extension in Bin2 which the yeast protein lacks argues that these proteins are not homologous. Bin2 sequences did not give clear insight into its function. A distant similarity was seen between the central domains of Bin2 and Daxx, but the significance of this relationship is uncertain. Both Daxx and certain Bin1 isoforms appear to influence cell survival decisions at some level but the mechanisms involved are currently obscure (Elliott *et al.,* 2000; Prendergast, 1999). Daxx has a physiological role in the control of programmed cell death (Michaelson *et al.,* 1999) and it interacts with proteins that influence death decisions, such as the Jun kinase regulator apoptosis-signaling kinase-1 and the tumor-suppressor Pml (Chang *et al.,* 1998; Quignon *et al.,* 1998; Torii *et al.,* 1999; Wang *et al.,* 1998). Similarly, ectopic complementation of the frequent deficiencies of Bin1 in cancer cells leads to engagement of a programmed cell death process (Ge *et al.,* 1999, 2000a). We did not observe any effects of Bin2 on cell survival, but further investigation to assess this connection may be worthwhile, insofar as it would be consistent with the role of certain Bin1 isoforms in cancer cells as well as with the role of Rvs proteins in yeast survival under stress conditions.

The human Bin2 gene mapped to the long arm of chromosome 4, at position 4q22.1, and Bin2 was expressed primarily in hematopoietic cells. The 4q22.1 region has been reported to be frequently disrupted in hepatocarcinoma and breast cancer (Rashid *et al.,* 1999; Schwendel *et al.,* 1998; Tirkkonen *et al.,* 1997; Yeh *et al.,* 1996). However, while we have obtained some results consistent with aberrant organization of the Bin2 gene in hepatoma cell lines, polymorphism cannot be ruled out, and we did not see any effect of ectopic Bin2 on the growth of hepatoma cells. Therefore, whether Bin2 losses or inactivation are relevant to malignancy is unclear. In contrast, a functional role for Bin2 in hematopoietic cells was supported by the finding that Bin2 expression is upregulated during granulocytic differentiation. In future work, it will be important to examine expression further by *in situ* methods to determine the range of potential Bin2 functions.

We found that Bin2 formed a stable biochemical complex with Bin1 but not with amphiphysin. Similar complexes have been reported between the BAR proteins Rvs161 and Rvs167 in yeast and amphiphysin and Bin1 in mammalian cells (Navarro *et al.,* 1997; Wigge *et al.,* 1997). The finding that amphiphysin did not interact with Bin2 is notable for several reasons. First, it supports the notion that Bin2 may not function in endocytosis. Second, it corroborates other evidence that BAR proteins including amphiphysin and Bin1 have unique functions, despite their structural similarities, insofar as Bin2 binds only Bin1. Third, this finding is consistent with observations in yeast that argue that BAR domains have overlapping but distinct func-

tions (Sivadon *et al.,* 1997). Finally, it suggests that BAR domains have specific oligomerization potentials, therefore raising the possibility that mammalian BAR protein functions are varied by combinatorial interactions. Given that amphiphysin and Bin2 are essentially tissue-restricted in expression, and possibly nonoverlapping, it is tempting to speculate that the function of Bin1 is differentially affected in tissues by coexpression with amphiphysin or Bin2.

We observed that Bin2–Bin1 association required N-terminal sequences within the BAR domain that include one of its two most highly conserved segments (aa 23–45). This segment is predicted to assume helical configuration, so it may participate in mediating association. In contrast, a second strongly conserved region of the BAR domain which is located in the C-terminal part (centered on aa 143–148) was dispensable for interaction, implying that it has a function other than mediating BAR protein oligomerization. Consistent with this likelihood, the C-terminal part of BAR in Bin1 is crucial for its tumor suppressor properties (Elliott *et al.,* 1999). While Bin2 is identical to Bin1 within this segment, it did not exhibit antiproliferative properties like Bin1. This difference in activity might reflect structural differences with Bin1 outside of the BAR domain, a possibility that could also be addressed by domain-swapping experiments. In future work, it will be important to define the physiological function of Bin2 by gene deletion in mice and to learn how Bin1 function is influenced by Bin2 interaction.

ACKNOWLEDGMENTS

We thank James DuHadaway and P. Scott Donover for technical assistance and P. McPherson for kindly providing full-length cDNAs for human amphiphysin and the brain-specific Bin1 splice isoform Bin1−10+12ABCD (also known as amphiphysin IIa). We are also grateful to A. Muller for critical comments on the manuscript. This work was supported in part by grants from the U.S. Army Breast Cancer and Prostate Cancer Research Programs (DAMD-96-1-6324 and PC970326). K.G. was supported by a fellowship from the Adler Foundation. G.C.P. is a Pew Scholar in the Biomedical Sciences.

REFERENCES

Antoine, J. C., Absi, L., Honnorat, J., Boulesteix, J. M., de Brouker, T., Vial, C., Butler, M., De Camilli, P., and Michel, D. (1999). Antiamphiphysin antibodies are associated with various paraneoplastic neurological syndromes and tumors. *Arch. Neurol.* **56:** 172–177.

Balguerie, A., Sivadon, P., Bonneu, M., and Aigle, M. (1999). Rvs167p, the budding yeast homolog of amphiphysin, colocalizes with actin patches. *J. Cell Sci.* **112:** 2529–2537.

Bauer, F., Urdaci, M., Aigle, M., and Crouzet, M. (1993). Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* **13:** 5070–5084.

Benmarah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. *J. Cell Sci.* **112:** 1303–1311.

Benmerah, A., Lamaze, C., Begue, B., Schmid, S. L., Dautry-Varsat, A., and Cerf-Bensussan, N. (1998). AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J. Cell Biol.* **140:** 1055–1062.

Breton, A. M., and Aigle, M. (1998). Genetic and functional relationship between Rvsp, myosin and actin in Saccharomyces cerevisiae. *Curr. Genet.* **34:** 280–286.

Brizzio, V., Gammie, A. E., and Rose, M. D. (1998). Rvs161p interacts with Fus2p to promote cell fusion in Saccharomyces cerevisiae. *J. Cell Biol.* **141:** 567–584.

Butler, M. H., David, C., Ochoa, G.-C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O., and De Camilli, P. (1997). Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/RVS family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of Ravier in brain and around T tubules in skeletal muscle. *J. Cell Biol.* **137:** 1355–1367.

Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998). Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science* **281:** 1860–1863.

Chen, C., and Okayama, H. (1987). High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:** 2745–2752.

Colwill, K., Field, D., Moore, L., Friesen, J., and Andrews, B. (1999). In vivo analysis of the domains of yeast Rvs167p suggests Rvs167p function is mediated through multiple protein interactions. *Genetics* **152:** 881–893.

Crouzet, M., Urdaci, M., Dulau, L., and Aigle, M. (1991). Yeast mutant affected for viability upon nutrient starvation: Characterization and cloning of the RVS161 gene. *Yeast* **7:** 727–743.

Dropcho, E. J. (1996). Antiamphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis. *Ann. Neurol.* **39:** 659–667.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Staller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G. C. (1999). Bin1 functionally interacts with Myc in cells and inhibits cell proliferation by multiple mechanisms. *Oncogene* **18:** 3564–3573.

Elliott, K., Ge, K., Du, W., and Prendergast, G. C. (2000). The c-Myc-interacting protein Bin1 activates a caspase-independent cell death process. *Oncogene,* in press.

Floyd, S., Butler, M. H., Cremona, O., David, C., Freyberg, Z., Zhang, X., Solimena, M., Tokunaga, A., Ishizu, H., Tsutsui, K., and de Camilli, P. (1999). Expression of amphiphysin I, an autoantigen of paraneoplastic neurological syndromes, in breast cancer. *Mol. Med.* **4:** 29–39.

Folli, F., Solimena, M., Cofiell, R., Austoni, M., Tallini, G., Fassetta, G., Bates, D., Cartlidge, N., Bottazzo, G. F., Piccolo, G., *et al.* (1993). Autoantibodies to a 128-kd synaptic protein in three women with the stiff-man syndrome and breast cancer. *N. Engl. J. Med.* **328:** 546–551.

Ge, K., DuHadaway, J., Du, W., Herlyn, M., Rodeck, U., and Prendergast, G. C. (1999). Mechanism for elimination of a tumor suppressor: Aberrant splicing of a brain-specific exon causes loss of function of Bin1 in melanoma. *Proc. Natl. Acad. Sci. USA* **96:** 9689–9694.

Ge, K., DuHadaway, J., Sakamuro, D., Wechsler-Reya, R., Reynolds, C., and Prendergast, G. C. (2000a). Losses of the tumor suppressor Bin1 in breast carcinoma are frequent and reflect deficits in a programmed cell death capacity. *Int. J. Cancer* **85:** 376–383.

Ge, K., Minhas, F., DuHadaway, J., Mao, N.-C., Wilson, D., Sakamuro, D., Buccafusca, R., Nelson, P., Malkowicz, S. B., Tomaszewski, J. T., and Prendergast, G. C. (2000b). Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma. *Int. J. Cancer* **86:** 155–161.

Huang, D., Patrick, G., Moffat, J., Tsai, L. H., and Andrews, B. (1999). Mammalian cdk5 is a functional homologue of the budding yeast Pho85. *Proc. Natl. Acad. Sci. USA* **96:** 14445–14450.

Kadlec, L., and Prendergast, A.-M. (1997). The amphiphysin-like protein 1 (ALP1) interacts functionally with the cABL tyrosine kinase and may play a role in cytoskeletal regulation. *Proc. Natl. Acad. Sci. USA* **94:** 12390–12395.

Lee, J., Colwill, K., Aneuliunas, V., Tennyson, C., Moore, L., Ho, Y., and Andrews, B. (1998). Interaction of yeast Rvs167 and Pho85 cyclin-dependent kinase complexes may link the cell cycle to the actin cytoskeleton. *Curr. Biol.* **8:** 1310–1321.

Lichte, B., Veh, R. W., Meyer, H. E., and Kilimann, M. W. (1992). Amphiphysin, a novel protein associated with synaptic vesicles. *EMBO J.* **11:** 2521–2530.

Lila, T., and Drubin, D. G. (1997). Evidence for physical and functional interactions among two Saccharomyces cerevisiae SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol. Biol. Cell* **8:** 367–385.

Mao, N. C., Steingrimsson, E., Ruiz, D. J. J., Wasserman, W., Copeland, N. G., Jenkins, N. A., and Prendergast, G. C. (1999). The murine Bin1 gene, which functions early in myogenic differentiation, defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. *Genomics* **56:** 51–58.

Michaelson, J. S., Bader, D., Kuo, F., Kozak, C., and Leder, P. (1999). Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. *Genes Dev.* **13:** 1918–1923.

Navarro, P., Durrens, P., and Aigle, M. (1997). Protein–protein interaction between the RVS161 and RVS167 gene products of Saccharomyces cerevisiae. *Biochim. Biophys. Acta* **1343:** 187–192.

Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., and Lerner, R. A. (1983). Generation of protein-reactive antibodies by short peptides is an event of high frequency: Implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA* **80:** 4949–4953.

Nishizawa, M., Kanaya, Y., and Toh-E., A. (1999). Mouse cyclin-dependent kinase (cdk) 5 is a functional homolog of a yeast ckd, pho85 kinase. *J. Biol. Chem.* **274:** 33859–33862.

Prendergast, G. C. (1999). Mechanisms of apoptosis by c-Myc. *Oncogene* **18:** 2966–2986.

Prendergast, G. C., and Cole, M. D. (1989). Posttranscriptional regulation of cellular gene expression by the c-myc oncogene. *Mol. Cell. Biol.* **9:** 124–134.

Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J. C., and de The, H. (1998). PML induces a novel caspase-independent death process. *Nat. Genet.* **20:** 259–265.

Ramjaun, A. R., and McPherson, P. S. (1998). Multiple amphiphysin II splice variants display differential clathrin binding: Identification of two distinct clathrin-binding sites. *J. Neurochem.* **70:** 2369–2376.

Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997). Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem.* **272:** 16700–16706.

Rashid, A., Wang, J. S., Qian, G. S., Lu, B. X., Hamilton, S. R., and Groopman, J. D. (1999). Genetic alterations in hepatocellular carcinomas: Association between loss of chromosome 4q and p53 gene mutations. *Br. J. Cancer* **80:** 59–66.

Sakamuro, D., Elliott, K., Wechsler-Reya, R., and Prendergast, G. C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nat. Genet.* **14:** 69–77.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Press, Cold Spring Harbor.

Schwendel, A., Richard, F., Langreck, H., Kaufmann, O., Lage, H., Winzer, K. J., Petersen, I., and Dietel, M. (1998). Chromosome alterations in breast carcinomas: Frequent involvement of DNA losses including chromosomes 4q and 21q. *Br. J. Cancer* **78:** 806–811.

Sivadon, P., Bauer, F., Aigle, M., and Crouzet, M. (1995). Actin cytoskeleton and budding pattern are altered in the yeast rvs161 mutant: The Rvs161 protein shares common domains with the brain protein amphiphysin. *Mol. Gen. Genet.* **246:** 485–495.

Sivadon, P., Crouzet, M., and Aigle, M. (1997). Functional assessment of the yeast Rvs161 and Rvs167 protein domains. *FEBS Lett.* **417:** 21–27.

Tirkkonen, M., Johannsson, O., Agnarsson, B. A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R. B., Borg, A., and Kallioniemi, O. P. (1997). Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res.* **57:** 1222–1227.

Torii, S., Egan, D. A., Evans, R. A., and Reed, J. C. (1999). Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *EMBO J.* **18:** 6037–6049.

Tsutsui, K., Maeda, Y., Tsutsui, K., Seki, S., and Tokunaga, A. (1997). cDNA cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. *Biochem. Biophys. Res. Commun.* **236:** 178–183.

Wang, Z. G., Ruggero, D., Ronchetti, S., Zhong, S., Gaboli, M., Rivi, R., and Pandolfi, P. P. (1998). PML is essential for multiple apoptotic pathways. *Nat. Genet.* **20:** 266–272.

Wechsler-Reya, R., Elliott, K., Herlyn, M., and Prendergast, G. C. (1997a). The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. *Cancer Res.* **57:** 3258–3263.

Wechsler-Reya, R., Elliott, K., and Prendergast, G. C. (1998). A role for the putative tumor suppressor Bin1 in muscle cell differentiation. *Mol. Cell. Biol.* **18:** 566–575.

Wechsler-Reya, R., Sakamuro, D., Zhang, J., Duhadaway, J., and Prendergast, G. C. (1997b). Structural analysis of the human BIN1 gene: Evidence for tissue-specific transcriptional regulation and alternate RNA splicing. *J. Biol. Chem.* **272:** 31453–31458.

Wigge, P., Kohler, K., Vallis, Y., Doyle, C. A., Owen, D., Hunt, S. P., and McMahon, H. T. (1997). Amphiphysin heterodimers: Potential role in clathrin-mediated endocytosis. *Mol. Biol. Cell* **8:** 2003–2015.

Wigge, P., and McMahon, H. T. (1998). The amphiphysin family of proteins and their role in endocytosis at the synapse. *Trends Neurosci.* **21:** 339–344.

Yeh, S. H., Chen, P. J., Lai, M. Y., and Chen, D. S. (1996). Allelic loss of chromosomes 4q and 16q in hepatocellular carcinoma: Association elevated alpha-fetoprotein production. *Gastroenterology* **110:** 184–192.

Cell Adhesion and Communication, 1999, Vol. 7, No. 2, pp. 99–110
Reprints available directly from the publisher
Photocopying permitted by license only

Bau, a Splice Form of Neurabin-I that Interacts with the Tumor Suppressor Bin1, Inhibits Malignant Cell Transformation

JAMES DUHADAWAY, FELICITY ROWE, KATHERINE ELLIOTT, NIEN-CHEN MAO[†] and GEORGE C. PRENDERGAST*

The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104-4268, USA

(Received 4 January 1999; In final form 7 January 1999)

Bin1 is a nucleocytoplasmic adaptor protein and tumor suppressor. A novel protein termed Bau was identified through its ability to interact with a region of Bin1 required to inhibit malignant cell transformation by certain oncogenes. Bau is a splice form of Neurabin-I, one of two related F-actin-binding proteins that are proposed to link cadherin-based cell–cell adhesion sites with the growth regulatory kinase $p70^{S6K}$. Bau lacks actin- and $p70^{S6K}$-binding domains found in Neurabin-I but includes coiled-coil domains that are part of its central domain as well as additional sequences not found in Neurabin-I. Interaction with Bin1 requires the presence of the U3 region which is alternately spliced in muscle cells. Bau localizes to the nucleus and cytosol. Like Bin1, Bau can suppress oncogene-mediated transformation and inhibit tumor cell growth. We suggest that Bau may link Bin1 to the Neurabin-I/$p70^{S6K}$ system in muscle and other cells, perhaps providing a mechanism to influence adhesion-dependent signals which affect cell fate.

Keywords: Transformation, tumor suppressor, Myc, adhesion

INTRODUCTION

Bin1 is an adaptor protein and tumor suppressor that was identified initially through its ability to interact with and inhibit malignant cell transformation by the c-Myc oncoprotein (Sakamuro *et al.*, 1996). Subsequent investigations have indicated complex roles for Bin1 in proliferation, apoptosis, and differentiation (Elliott *et al.*, 1999a,b; Sakamuro *et al.*, 1999; Wechsler-Reya *et al.*, 1998). The Bin1 gene undergoes complex patterns of alternate splicing, especially in neurons (Butler *et al.*, 1997; Wechsler-Reya *et al.*, 1997b). Different splice forms have been cloned, variously termed

* Corresponding author. Tel.: +215 898 3792. Fax: +215 898 2205. E-mail: prendergast@wistar.upenn.edu.
[†] Present address. DuPont Pharmaceuticals, Wilmington, DE 19880.

SH3P9, amphiphysin-like protein, and amphiphysin II, based on the presence of an SH3 domain, SH3-dependent interaction with the tyrosine kinase c-Abl, or structural relationship with the neuron-specific protein amphiphysin (Butler *et al.*, 1997; Kadlec and Pendergast, 1997; Ramjaun *et al.*, 1997; Sparks *et al.*, 1996; Tsutsui *et al.*, 1997; Wigge *et al.*, 1997a). All the splice forms of Bin1 include N- and C-terminal regions related to amphiphysin, which is a paraneoplastic autoimmune antigen implicated in receptor-mediated neuronal endocytosis and synaptic function (David *et al.*, 1994; 1996; Dropcho, 1996; Wigge *et al.*, 1997b). Brain-specific splice forms of Bin1 termed amphiphysin II interact with amphiphysin and have been implicated in endocytosis (Wigge *et al.*, 1997a,b). However, non-neuronal splice forms of Bin1 are unlikely to be involved in endocytosis because they lack sequences required for binding to clathrin and therefore localization to endocytotic vesicles (Ramjaun and McPherson, 1998). Bin1 is also related to RVS167 and RVS161, two negative regulators of the cell cycle in yeast which are implicated in cytoskeletal actin regulation, endocytosis, cell fusion, and karyogamy (Bauer *et al.*, 1993; Breton and Aigle, 1998; Brizzio *et al.*, 1998; Munn *et al.*, 1995). Taken together, investigations of this family of gene products, which we have termed BAR family proteins (Bin1/Amphiphysin/RVS-related proteins), suggests that they are nucleocytoplasmic adaptors that link cytosolic and nuclear events via a novel set of signal transduction pathway(s).

Bin1 inhibits malignant growth by c-Myc but also by adenovirus E1A (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996). The latter effects are c-Myc-independent and dependent upon the U1 region of Bin1 (Elliott *et al.*, 1999b). An adjacent region of Bin1 termed U3 which is alternately spliced in muscle cells potentiates growth inhibition by Bin1 in those cells (Wechsler-Reya *et al.*, 1998). We performed a two hybrid screen to identify proteins which might mediate the U1/U3-dependent and c-Myc-independent growth inhibitory properties of Bin1. In this report we identify one such protein termed Bau (Bin1-Associated U1/U3-specific binding protein). Bau is a novel growth inhibitory protein and splice form of Neurabin-I, which associates with the growth regulatory kinase $p70^{S6K}$ and has been proposed to link cytoskeletal actin with cell–cell adhesion sites. Our findings suggest a link between Bin1 and the Neurabin-I/$p70^{S6K}$ system in cells.

MATERIALS AND METHODS

Two Hybrid Analysis

The two hybrid system, murine embryo cDNA library, and methodologies used to clone Bau have been described in detail elsewhere (Sakamuro *et al.*, 1996; Vojtek *et al.*, 1993). Briefly, the bait plasmids were marked with TRP1 and used lexA as a DNA binding component, and the library and prey plasmids were marked with LEU2 and used the herpes simplex virus VP16 as a transcriptional transactivating component. The 10.5 day embryo library was constructed with cDNA degraded by random DNAse I treatment to $\sim$0.5 kb, treated with Klenow enzyme, Not I linkered, and subcloned into the bait plasmid pVP16 (S. Hollenberg, unpublished data). This library was designed to identify protein modules which might be occluded in full-length polypeptides. Yeast strain L40 (MATa trp1-901 leu2-3,112 LYS2::$(lexAop)_4$-HIS3 URA3::$(lexAop)_8$-lacZ) was used for the assay (Vojtek *et al.*, 1993). One hundred interacting clones cured of the bait plasmid were tested for interaction by a mating strategy using the L40 derivative AMR70 (R. Sternglanz, unpublished data). Clones that complemented his auxotrophy and activated lexA-dependent expression of LacZ in L40 were considered positive for interaction, subcloned, and sequenced. Test baits in AMR70 included the original lexA-U1/U3 construct and a set of negative controls including no insert, lamin (Vojtek *et al.*, 1993), or the control peptide FTR-HPPVLTPPDQEVI derived from rat protein kinase C β2 (the latter which controls for non-specific peptide interactions, e.g. peptidases; G.C.P. and K. Koblan, unpublished results).

Plasmid Construction and DNA Sequencing

Yeast two hybrid and *E. coli* glutathione-S-transferase vectors summarized in Figs. 3 and 4 were constructed by standard PCR and recombinant DNA technology (cloning details are omitted to save space but are available from G.C.P.). The clone #100 cDNA isolated by two hybrid assay was used as a probe to clone a ~1 kb Bau cDNA from a murine embryo λZAPII cDNA library (Stratagene) using standard methods. The complete primary structure of this cDNA was determined on an automated DNA sequencer and assembled and analyzed with MacVector software (Kodak). DNA database comparisons were performed using BLAST client software. For expression in mammalian cells, clone #100 and Bau cDNAs were subcloned into the cytomegalovirus (CMV) enhancer/promoter-driven vectors pcDNA3 and pDNA3.1-Myc-His-A (Invitrogen), generating CMV-Bau and CMV-BBD (clone #100). Clone #100 cDNA was modified with a Kozak translation initiation sequence by shuttling it first into pATG (Sakamuro *et al.*, 1996). The Bin1 vector CMV-Bin1 and the oncogenic H-Ras vector pT22 have been described (Land *et al.*, 1983; Sakamuro *et al.*, 1996). The adenovirus E1A vector p1A/neo and the SV40 T antigen vector neoCMV T were gifts from N. Kohl.

Northern Analysis

Total cytoplasmic RNA was isolated and analyzed essentially as described (Prendergast and Cole, 1989). Total RNA from murine embryo and adult tissues was a gift from L. Benjamin. RNAs were fractionated on formaldehyde gels and Northern blots were hybridized to [32P]-labeled clone #100 cDNA probes (Church and Gilbert, 1984).

Immunoprecipitation and Immunofluorescence

A Bau-specific mouse monoclonal antibody was raised to a GST-clone #100 immunogen by the Wistar Hybridoma Core Facility. Briefly, BALB/c mice were immunized with GST-clone #100 and hybridomas were generated using the nonsecreting murine myeloma T3X63Ag8.Sp2/0 (Koprowski *et al.*, 1979). Hybridoma supernatants were screened by ELISA using unfused GST as a negative control and specificity was confirmed by immunoprecipitation and Western blotting of recombinant proteins. One hybridoma producing IgG strongly positive for the immunogen was used in this study. Transiently transfected COS cells were metabolically labeled for 4 h in DMEM media lacking methionine and cysteine (Life Technologies) with 75–125 μCi/mL EXPRESS labeling reagent (NEN) and cell extracts were prepared in NP40 buffer containing the protease inhibitors leupeptin. aprotinin, phenylmethylsulfonyl fluoride, and antipain (Harlow and Lane, 1988). Immunoprecipitation was performed using 100 μL hybridoma supernatant followed by SDS-PAGE and fluorography as described (Sakamuro *et al.*, 1996). For indirect immunofluorescence, COS cells were seeded onto glass cover slips in 6 cm dishes and the next day transfected overnight with 4 μg CMV-Bau or pcDNA3 vector. Two days later cells were washed and processed for immunofluorescence essentially as described (Prendergast and Ziff, 1991; Wechsler-Reya *et al.*, 1997a) using 100 μL of anti-Bau IgG and a 1 : 1000 dilution of fluorescein-conjugated anti-rabbit IgG (Cappel) as the secondary antibody. Stained cells were photographed using a Leitz fluorescence microscope apparatus.

GST Pulldown Assay

Glutathione-S-transferase (GST) fusion polypeptides were expressed and purified from *E. coli* cell extracts on glutathione-Sepharose (Pharmacia) using protocols supplied by the vendor. ^{35}S-methionine labeled polypeptides were generated by *in vitro* translation of CMV-Bau or CMV-BBD (clone #100) using TNT rabbit reticulocyte lysates (Promega). Approximately 5 μg of GST protein and 10 μL of an IVT reaction were added to 0.5 mL binding buffer (10 mM TrisHCl pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.5% NP40), incubated 1 h at 4°C on a nutator shaker, and washed four times

with binding buffer. Bound peptides were eluted by boiling in SDS gel loading buffer and then analyzed by SDS-PAGE and fluorography.

Tissue Culture

Cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Life Technologies) and 50 U/mL each penicillin and streptomycin (Fisher). Primary rat embryo fibroblasts (REFs; Whittaker Bioproducts) were cultured and transfected as described (Prendergast *et al.*, 1992). Briefly, secondary passage REFs seeded into 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method (Chen and Okayama, 1987) with 5 μg each p1A/neo or neoCMV T plus 10 μg of the CMV vector indicated, passaged 1 : 5 the next day, and then fed with normal growth media until foci were scored by methanol fixation and crystal violet staining 12–14 days later. Colony formation assays in HepG2 were performed as described using 2 μg of the vectors indicated. Cells were cultured in growth media containing 0.75 mg/mL G418 (Life Technologies) to select for the neomycin resistance gene cassette on the CMV vector. Colonies were scored by methanol fixation and crystal violet staining ~3 weeks after transfection.

RESULTS

To identify U1/U3-binding proteins a 55 aa non-transactivating fragment of Bin1 that included the U1 and U3 regions (aa 214–269) (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996) was used as "bait" in a lexA/VP16-based two hybrid screen (see Materials and Methods). One hundred clones from a set of 425 U1/U3-interacting candidates obtained in a screen of $\sim 7 \times 10^7$ cDNAs from a murine 10.5 day embryo library were analyzed by a mating strategy as described previously (Sakamuro *et al.*, 1996). Nonspecific interactions with empty vector, lamin, or a control peptide derived from protein kinase C (PKC) peptide were discarded. Two sequences were represented among 7 clones obtained that were strongly positive and specific for U1/U3 interaction, as indicated by complementation of his auxotrophy and activation of lacZ activity in L40 cells (data not shown). Two of the clones were novel and had different fusion junctions with lexA. The complete sequence of one of these, clone #100, included a 146 aa open reading frame (ORF) that was unrelated to known sequences at the time of cloning. Preliminary Northern analysis usine clone #100 as a probe showed hybridization to a ~10 kb brain- and muscle-specific RNA and a more widely expressed ~1.3 kb RNA. Nakanishi *et al.* (1997) subsequently reported the ~10 kb message as Neurabin-I but did not refer to the smaller message. Bin1 is abundant in brain and muscle but is also more widely expressed like Bau so we proceeded to characterize a full length cDNA for the smaller ~1.3 kb message, which was isolated by standard methods from a lambda phage murine E10.5 embryo library. Figure 1 shows the structure of the protein encoded by this cDNA, termed Bau (for Bin1-Associated U1/U3-specific binding protein), and illustrates its relationship to Neurabin-I. Bau comprised a 293 aa ORF with a predicted MW of 34080. By inference the Bin1-binding domain (BBD) as encompassed by clone #100 was located between aa 66–207 of Bau. The region of identity between Bau and Neurabin-I encompassed two helical regions predicted to participate in coiled-coil interactions (aa 739–779 and aa 787–834, marked helices *b* and *c* in Fig. 1, respectively). Both were also included within the BBD as defined by clone #100. Interestingly, while aa 1–255 in Bau was identical to rat Neurabin-I (aa 665–920) the C-terminal region from aa 256–293 diverged (hatched segment in Fig. 1). Taken together the results argued that the ~1.3 kb message encoding Bau was an alternately spliced form of Neurabin-I.

Bau is More Widely Expressed than Neurabin-I and Localizes to the Nucleus and Cytosol

Northern analysis of a panel of embryonic and adult mouse tissues showed that Bau expression

```
Bau          MFSPSDLDTSKLSHKFKELQIKHAVTEAEIQKLKTKLQASENEKVRWELE   50

Bau          KNQLQQNIEENKERMLKLESYWIEAQTLCHTVNEHLKETQSQYQALEKKY  100
clone 100    ..........KERMLKLESYWIEARTLCHTVNEHLKETQSQYQALEKKY

Bau          NKAKKLIKDFQQKELDFIKRQEVERKKREEVEKAHLLEVQGLQVRIRDLE  150
clone 100    NKAKKLIKDF.QKELDFIKRCEVERKKREEVEKAHLLEVQGLQVRIRELE

Bau          AEVFRLLKQNGTQVNNNNNNIFERRPSPGEVSKGDTMENVEVKQTSCQDGL  200
clone 100    AEVFRLLKQNGTQVNNNNNIFERRPPPGEVSKGDTMENVEVKQTSCQDGL

Bau          SQDLNEAVPETERLDSKALKTRAQLSVKNRRQRPTRTRLYDSVSSTDGED  250
clone 100    SQDLNEA.....................................

Bau          SLERKVSTLNGWQTLAECRCPPVYLLNVIAVLLICALLGRKSP*         293
```

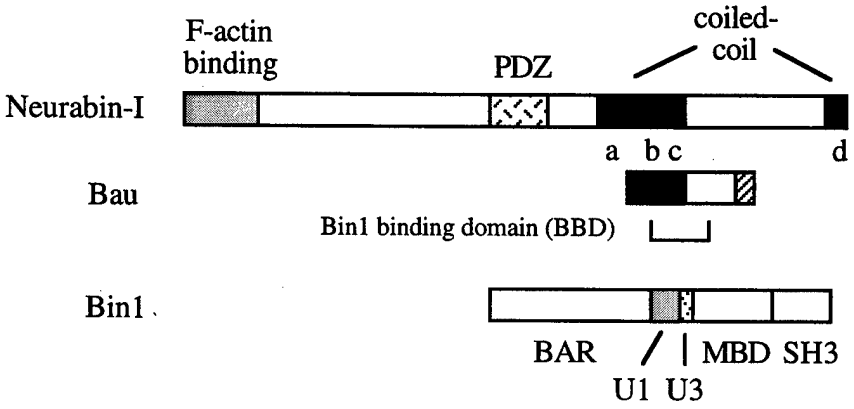


FIGURE 1 *Bau structure.* Top, amino acid sequence of Bau and BBD defined by clone 100 (partial two hybrid cDNA). The N-terminal sequences that are underlined are shared with Neurabin-I (Nakanishi *et al.*, 1997); the remaining C-terminal sequences are unique to Bau. Bottom, cartoon depicting Neurabin-I, Bau, and Bin1 structure. Features of Neurabin-I including F-actin-binding and PDZ domains and *a, b, c, d* helices predicted to participate in coiled-coil interactions are taken from Nakanishi *et al.*, 1997. The BBD defined by clone 100 encompasses the *b, c* coiled-coil helices. The hatched bar in Bau depicts unique sequences not found in Neurabin-I. The U1 and U3 sequences in the central region of Bin1 used as "bait" in the two hybrid screen are shaded and stippled, respectively. BAR, Bin1/amphiphysin/RVS-related domain; MBD, Myc-binding domain; SH3, Src homology-3 domain.

overlapped with Neurabin-I but was more ubiquitous (see Fig. 2A). A testis-specific band of ~2.5 kb was also hybridized by the Bau cDNA (Fig. 2A, right arrowhead). Bau was expressed in human diploid WI-38 fibroblasts lacking detectable Neurabin-I but similar to Bin1 (Sakamuro *et al.*, 1996) its expression varied in malignant human cells (see Fig. 2B). Bin1 is localized in the nucleus but also in the cytoplasm of certain cell types so it was of interest to determine Bau localization. COS cells were transiently transfected with expression vectors for Bau, clone #100, or Bin1 and cell extracts were subjected to immunoprecipitation with a monoclonal antibody (mAb) raised to Bau (see Fig. 2C). A ~45 or ~23 kD polypeptide was precipitated from cells transfected with Bau or clone #100, respectively. Both were larger than the ~34 and ~17 kD polypeptides predicted on the basis of amino acid sequence but each was specific, insofar as Bin1 was not precipitated by the anti-Bau mAb

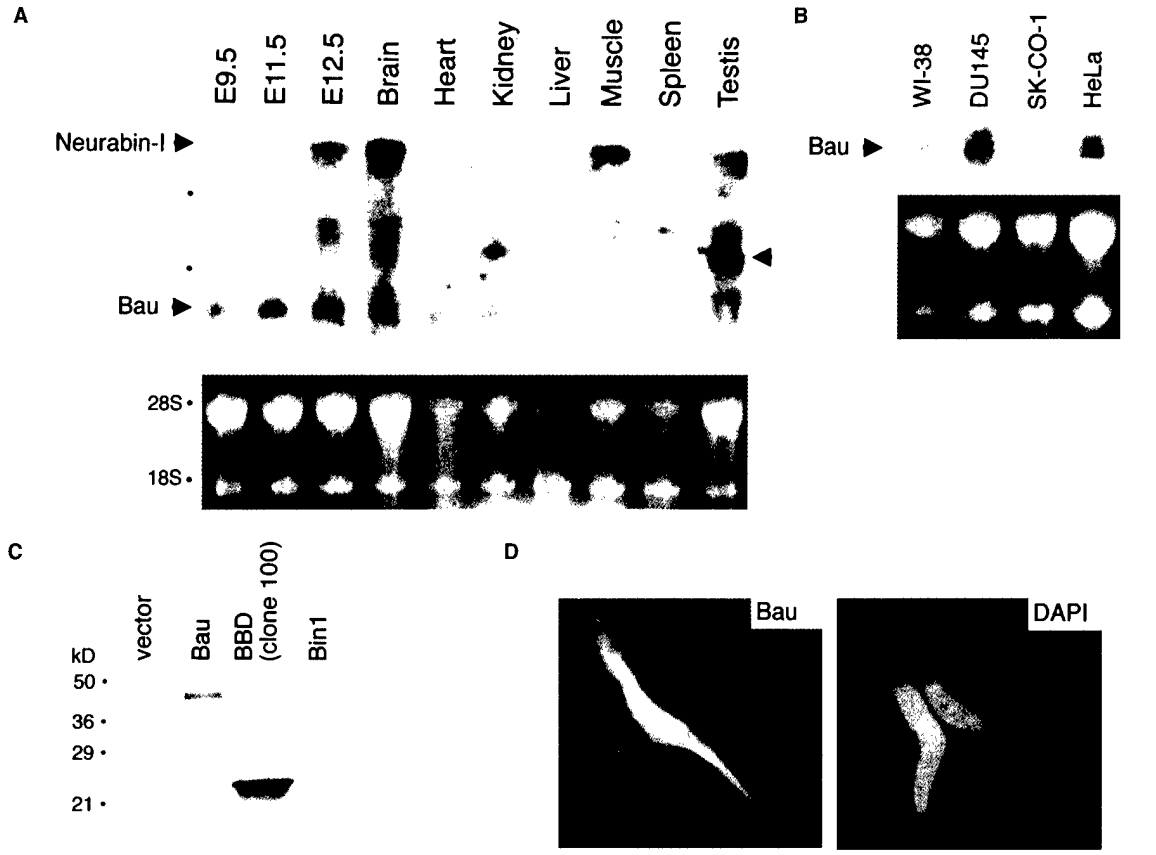


FIGURE 2 *Bau expression and localization.* (A) Northern analysis of mouse tissues. Northern blot of total cytoplasmic RNA from embryonic and adult mouse tissues was hybridized with a radiolabeled murine clone 100 probe. The large Neurabin-I and small Bau messages are indicated. The asterisk indicates an additional Neurabin-I/Bau-related message in adult kidney and testis. The Northern gel was photographed before transfer to document equivalent RNA loading. (B) Northern analysis of human cells. Northern blot of total cytoplasmic RNA from WI-38 diploid fibroblasts, DU145 prostate carcinoma, SK-CO-1 colon carcinoma, and HeLa cervix carcinoma cells was hybridized as above. (C) Immunoprecipitation of recombinant Bau and BBD (clone 100). Extracts from COS cells transfected with the CMV vector indicated and metabolically labeled with ^{35}S-methionine were subjected to immunoprecipitation with an anti-Bau monoclonal antibody. Immunoprecipitates were fractionated by SDS-PAGE and the gel was dried and fluorographed. (D) COS immunofluorescence. COS cells were transfected with CMV-Bau and processed for indirect immunofluorescence using anti-Bau monoclonal antibody. Nuclei were identified by DAPI counterstain.

from COS cells transfected with a vector for that protein and little background was present. Further immunoprecipitations with the same mAb identified a ~45 kD band in differentiated C2C12 myoblasts (data not shown), a model for analysis of Bin1 function (Wechsler-Reya *et al.*, 1998), consistent with its identity as Bau and suggesting that it had an aberrant mobility on SDS gels. Indirect immunofluorescence was performed following similar transfection of COS cells. Bau was found to localize in both the nucleus and cytoplasm with a slight preference for the former in cells where its expression was elevated. The localization results supported the possibility that Bau and Bin1 may interact in cells.

Bau Interacts with Bin1

Association between Bau and the Bin1 U1/U3 region was documented by two hybrid assay and GST pulldown experiments (see Fig. 3). In two hybrid assays, while interaction was strongest with

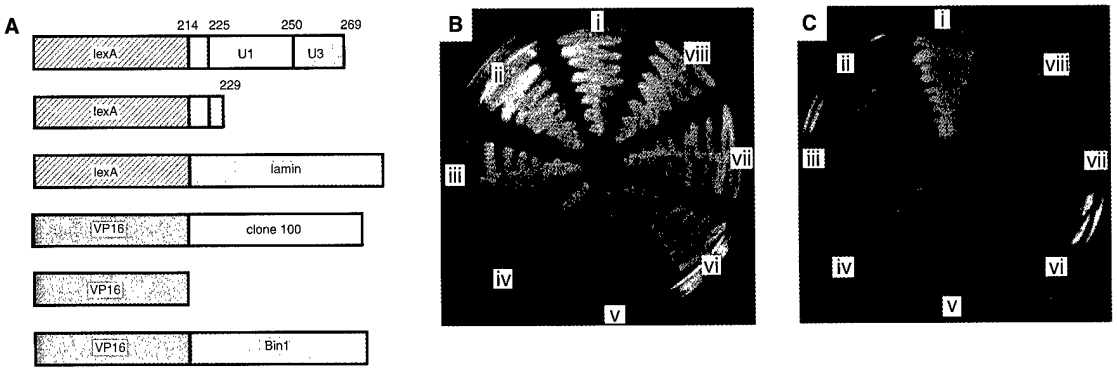


FIGURE 3 *Two hybrid analysis.* (A) Interaction vectors. LexA-based "bait" plasmids marked with TRP1 and VP16-based "prey" plasmids marked with LEU2 used in the experiment are shown. (B) Growth on Y_c-trp/leu plates. Complementation of trp/leu auxotrophy in L40 yeast cells documents presence of "bait" and "prey" interaction plasmids. (i) lexA-U1/U3 + VP16-clone 100; (ii) lexA-U1/U3 + VP16-Bin1; (iii) lexA-U1/U3 + VP16; (iv) lexA-U1/U3Δ229–269 + VP16-clone 100; (v) lexA-U1/U3Δ229–269 + VP16; (vi) lexA-lamin + VP16-clone 100; (vii) lexA-lamin + VP16-Bin; (viii) lexA-lamin + VP16. (C) Growth on Y_c-trp/leu/his plates. Interaction between U1/U3 and clone 100 implied by complementation of his auxotrophy in L40 cells expressing lexA-U1/U3 and VP16-clone 100 (i). Weak interaction was still detected with U1/U3Δ229–269 implying that aa 214–229 in this determinant in the BAR-C domain of Bin1 (Elliott *et al.*, 1999b) contributes to binding.

the full U1/U3 bait, a weaker but specific interaction persisted in the absence of U1/U3 if aa 214–229 from the highly conserved BAR-C region were retained. This suggested that BAR-C included a determinant that contributes to Bau binding. GST pulldown experiments confirmed that the interaction was due to direct biochemical association and revealed that both U1 and U3 were required for efficient Bau binding (see Fig. 4). U3 is spliced specifically into Bin1 during differentiation of muscle (Wechsler-Reya *et al.*, 1998) so this implied that Bin1–Bau interaction may be most prominent in that tissue.

Bau Inhibits Malignant Cell Growth

Bin1 is a tumor suppressor that can inhibit malignant cell transformation by c-Myc and adenovirus E1A but not by SV40 T antigen (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996). As indicated above, U1 had been of interest since its integrity was necessary for Bin1 to suppress transformation by E1A but not by c-Myc (Elliott *et al.*, 1999b). To determine if Bau acted similarly to Bin1 we asked whether it also inhibited malignant cell transformation, using the standard rat embryo fibroblast (REF) Ras cotransformation assay. Similar to Bin1, Bau suppressed transformation by E1A but not by T antigen and the BBD (clone #100) was sufficient for this effect (see Fig. 5). Additional experiments indicated that Bau could augment inhibition by Bin1 but that Bau was dispensable for the latter, since antisense Bau had no effect on the ability of Bin1 to suppress E1A transformation (data not shown). Nevertheless, a link between Bin1 U1 and Bau was supported by observations that neither Bau nor BBD significantly affected transformation by c-Myc (data not shown). Thus, Bau mimicked the ability of Bin1 to suppress the oncogenic properties of E1A but not c-Myc. To determine if Bau also had tumor suppressor effects we tested its effects on HepG2 growth, using a colony formation experiment used to assay Bin1 activity (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996). In this assay, Bau and BBD exhibited growth inhibitory activity that was significant but modest relative to Bin1 (see Fig. 6). The limited activity displayed by Bau was correlated with its ability to block the oncogenic effects of E1A but not c-Myc.

DISCUSSION

In this study we characterized a novel splice form of the Neurabin-I gene termed Bau and showed

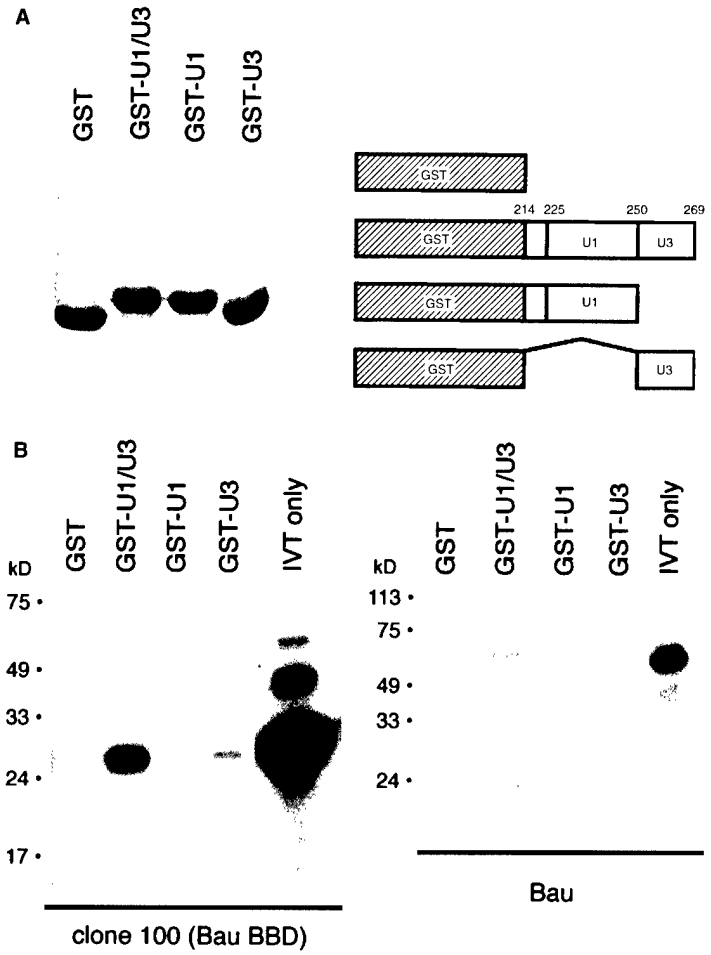


FIGURE 4 *Biochemical interaction.* (A) Production of GST fusion proteins. GST fusion proteins produced in *E. coli* by standard methods were fractionated by SDS-PAGE and the gel was fixed and stained with Coomassie blue. Protein structures are illustrated to the right of the gel photo. (B) Binding assay. The GST fusion proteins indicated were incubated in binding buffer with ^{35}S-methionine-labeled clone 100 (Bau BBD) or full-length Bau generated by *in vitro* translation. Protein bound following four washes with binding buffer was eluted and analyzed by SDS-PAGE and fluorography.

that it can interact with Bin1 and inhibit the oncogenic properties of the E1A oncoprotein. Bau interaction depended on the U1 region that is required for the c-Myc-independent growth inhibitory properties of Bin1 (Elliott *et al.*, 1999b). Although Bau is a splice variant of Neurabin-I that included divergent sequences, the region of Bau required for Bin1 interaction was entirely included in Neurabin-I. Thus, it is quite conceivable that Bin1 interacts with Neurabin-I in cells. Neurabin-I was initially identified as an F-actin-binding protein that is abundant in neurons and concentrated at synapses (Nakanishi *et al.*, 1997). A subsequent study confirmed concentration at synaptosomes and demonstrated that Neurabin-I interacts via its PDZ domain with S6 kinase ($p70^{S6K}$), an important regulator of translation and cell growth (Burnett *et al.*, 1998). An isoform of Neurabin-I that is ubiquitously expressed is termed Spinophilin, Neurabin-II, or KS5 (Allen *et al.*, 1997; Satoh *et al.*, 1998; Suh *et al.*, 1998). Spinophilin is a protein phosphatase-1-binding protein that localizes to dendritie spines

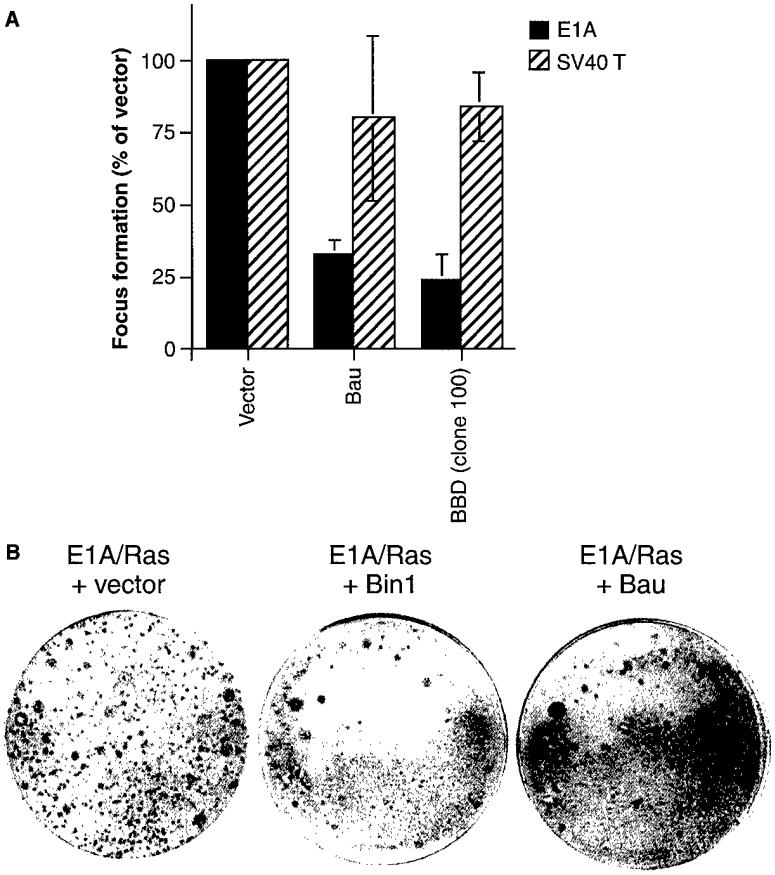


FIGURE 5 *Bau suppresses malignant cell transformation by adenovirus E1A.* (A) REF focus formation assay. Rat embryo fibroblasts were cotransfected with 5 μg vectors for adenovirus E1A or SV40 T antigen and oncogenic H-Ras as described (Prendergast *et al.*, 1992) plus 10 μg of the indicated CMV vectors. Transformed cell foci was scored 12–16 days later. The results are depicted as a proportion of the foci generated in the presence of vector only. The mean and standard error determined from four trials is shown. (B) Representative results. A set of dishes from one trial that included Bin1 as a positive control for suppression is shown.

(Allen *et al.*, 1997) in neurons and to adherens junctions in nonneuronal cells (Satoh *et al.*, 1998). Like Neurabin-I, Spinophilin has a PDZ domain that mediates interaction with $p70^{S6K}$ (Allen *et al.*, 1997; Burnett *et al.*, 1998; Satoh *et al.*, 1998). Both isoforms have the *a*, *b*, *c* helices implicated in coiled-coil interactions (see Fig. 1) that have been proposed to mediate homodimer or heterodimer formation (Burnett *et al.*, 1998). Bau includes the *b*, *c* helices so it may competitively regulate Neurabin interactions, if they occur. Similarly, since it is conceivable that Bin1 may interact with Neurabin isoforms, then Bin1 may also regulate Neurabin dimerization and perhaps function. This is potentially of interest in neurons, where neuron-specific splice forms of Bin1 have been implicated in endocytosis and synaptic function (David *et al.*, 1996; Ramjaun and McPherson, 1998; Wigge *et al.*, 1997a,b). However, since strong Bau interaction required the presence of the U3 region of Bin1, which is only alternately spliced into Bin1 in skeletal muscle, then interactions between Bin1 and Bau or the Neurabins may only be important in muscle or as yet unidentified tissues where U3 is expressed.

A link between Bin1 and the Neurabin/$p70^{S6K}$ system is potentially interesting because of the

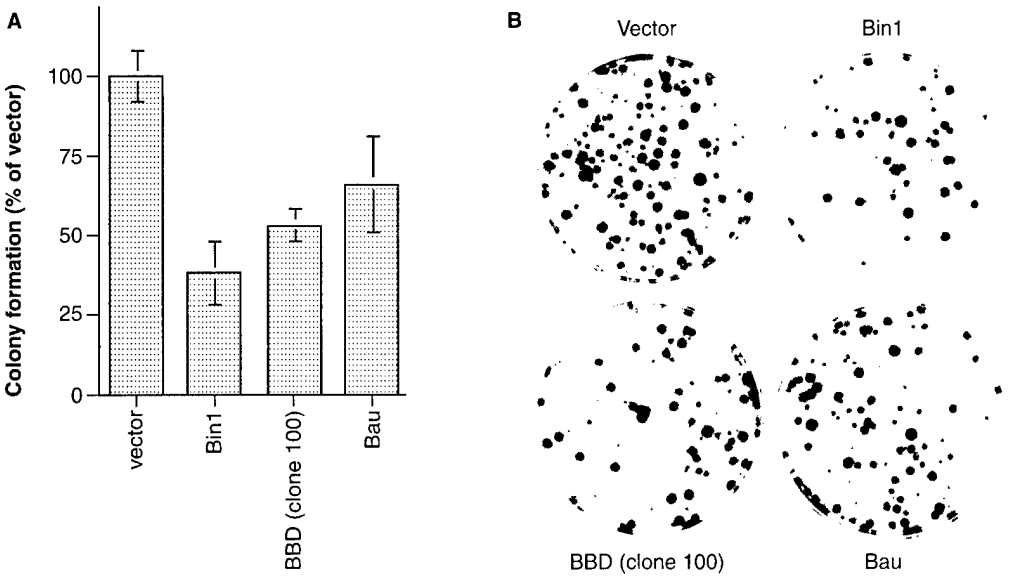


FIGURE 6 *Effect of Bau on tumor cell proliferation.* (A) HepG2 colony formation assay. HepG2 hepatoma cells were transfected with the CMV vectors indicated as described (Sakamuro *et al.*, 1996) and the neomycin resistance cassette on each vector was selected for by culturing cells in G418. Drug-resistant colonies were scored by fixation and crystal violet staining ~3 week posttransfection. Bin1 was included as a positive control for suppression. The results are depicted as a proportion of the colonies generated by vector only. The mean and standard error determined from three trials is shown. (B) Representative results. A set of dishes from one three trials is shown.

apparent roles of Bin1 in contributing to the determination of different cell fates following cell cycle exit. For example, in myoblasts that can exit the cell cycle appropriately following serum deprival, Bin1 contributes to this process and promotes differentiation (Wechsler-Reya *et al.*, 1998). In contrast, if cells cannot exit the cell cycle due to the enforced expression of c-Myc, then Bin1 is required for abortive apoptosis elicited by serum deprival (Sakamuro *et al.*, 1999). Given its properties as an adaptor that could participate in cell fate decisions, the findings of this report raise the possibility of some role for the Neurabins in cell fate. In support of this likelihood, it was recently shown that the rat homolog of the *C. elegans* cell fate gene product *Lin-10* associates in cells with Spinophilin/Neurabin-II (Ide *et al.*, 1998). It is tempting to speculate how Bin1 may coordinate the activities of nuclear growth regulatory proteins such as c-Myc and Rb/E2F (one of the main targets of E1A action), with important cytosolic growth regulatory signals controlled by $p70^{S6K}$ and the cell–cell attachment signaling pathways that have been proposed for the Neurabins. As an adaptor protein Bin1 may provide a mechanism to link and coordinate the activities of these diverse systems. In future work, it will be important to explore the potential physiological connections between Bin1, Bau, and the Neurabin/$p70^{S6K}$ system.

Acknowledgments

We thank Roberto Buccafusca for expert technical assistance. We acknowledge support from the Wistar Cancer Core grant (CA10815) and the Wistar Hybridoma, Oligonucleotide Synthesis, and DNA Sequencing Core Facilities. G.C.P. is supported by grants from the US Army Breast Cancer Research Program (DAMD17-96-1-6324) and the American Cancer Society (CN-160) and awards from CaPCURE. K.E. was supported by an NIH training grant. G.C.P. is a Pew Scholar in the Biomedical Sciences.

References

Allen, P.B., Ouimet, C.C. and Greengard, P. (1997) Spinophilin, a novel protein phosphatase-1 binding protein localized to dendritic spines. *Proc. Natl. Acad. Sci. USA* **94**, 9956–9961.

Bauer, F., Urdaci, M., Aigle, M. and Crouzet, M. (1993) Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* **13**, 5070–5084.

Breton, A.M. and Aigle, M. (1998) Genetic and functional relationship between Rvsp, myosin and actin in *Saccharomyces cerevisiae*. *Curr. Genet.* **34**, 280–286.

Brizzio, V., Gammie, A.E. and Rose, M.D. (1998) Rvs161p interacts with Fus2p to promote cell fusion in *Saccharomyces cerevisiae*. *J. Cell Biol.* **141**, 567–584.

Burnett, P.E., Blackshaw, S., Lai, M.M., Qureshi, I.A., Burnett, A.F., Sabatini, D.M. and Snyder, S.H. (1998) Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. *Proc. Natl. Acad. Sci. USA* **95**, 8351–8356.

Butler, M.H., David, C., Ochoa, G.-C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O. and De Camilli, P. (1997) Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/RVS family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of Ravier in brain and around T tubules in skeletal muscle. *J. Cell Biol.* **137**, 1355–1367.

Chen, C. and Okayama, H. (1987) High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745–2752.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.

David, C., McPherson, P.S., Mundigl, O. and de Camilli, P. (1996) A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA* **93**, 331–335.

David, C., Solimena, M. and De Camilli, P. (1994) Autoimmunity in Stiff-Man Syndrome with breast cancer is targeted to the C-terminal regulation of human amphiphysin, a protein similar to the yeast proteins. Rvs161 and Rvs167. *FEBS Lett.* **351**, 73–79.

Dropcho, E.J. (1996) Antiamphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis. *Ann. Neurol.* **39**, 659–667.

Elliott, K., Ge, K. and Prendergast, G.C. (1999a) Bin1 activates an apoptosis program in malignant cells that is independent of p53 and caspases. Manuscript in preparation.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Staller, P., Gaubatz, S., Zhong, H., Prochownik, E., Eilers, M. and Prendergast, G.C. (1999b) Bin1 functionally interacts with Myc in cells and inhibits cell proliferation by multiple mechanisms. *Oncogene* in press.

Ide, N., Hata, Y., Hirao, K., Irie, M., Deguchi, M., Yao, I., Satoh, A., Wada, M., Takahashi, K., Nakanishi, H. and Takai, Y. (1998) Interaction of rat Lin-10 with brain-enriched F-actin-binding protein, Neurabin-II/Spinophilin. *Biochem. Biophys. Res. Comm.* **244**, 258–262.

Kadlec, L. and Pendergast, A.-M. (1997) The amphiphysin-like protein 1 (ALP1) interacts functionally with the cABL tyrosine kinase and may play a role in cytoskeletal regulation. *Proc. Natl. Acad. Sci. USA* **94**, 12390–12395.

Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D. and Fuhrer, P. (1979) Colorectal carcinoma antigens detected by hybridoma antibodies. *Som. Cell Genet.* **5**, 957–972.

Land, H., Parada, L.F. and Weinberg, R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**, 596–602.

Munn, A.L., Stevenson, B.J., Geli, M.I. and Riezman, H. (1995) end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **6**, 1721–1742.

Nakanishi, H., Obaishi, H., Satoh, A., Wada, M., Mandai, K., Satoh, K., Nishioka, H., Matsuura, Y., Mizoghuchi, A. and Takai, Y. (1997) Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation. *J. Cell Biol.* **139**, 951–961.

Prendergast, G.C. and Cole, M.D. (1989) Posttranscriptional regulation of cellular gene expression by the c-myc, oncogene. *Mol. Cell. Biol.* **9**, 124–134.

Prendergast, G.C., Hopewell, R., Gorham, B. and Ziff, E.B. (1992) Biphasic effect of Max on Myc transformation activity and dependence on N- and C-terminal Max functions. *Genes Dev.* **6**, 2429–2439.

Prendergast, G.C. and Ziff, E.B. (1991) Mbh1: A novel gelsolin/severin-related protein which binds actin *in vitro* and exhibits nuclear localization *in vitro*. *EMBO J.* **10**, 757–766.

Ramjaun, A.R. and McPherson, P.S. (1998) Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites. *J. Neurochem.* **70**, 2369–2376.

Ramjaun, A.R., Micheva, K.D., Bouchelet, I. and McPherson, P.S. (1997) Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem.* **272**, 16700–16706.

Sakamuro, D., DuHadaway, J., Ewert, D. and Prendergast, G.C. (1999) A necessary role for Bin1 in Myc-mediated apoptosis. Manuscript submitted.

Sakamuro, D., Elliott, K., Wechsler-Reva, R. and Prendergast, G.C. (1996) BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nature Genet.* **14**, 69–77.

Satoh, A., Nakanishi, H., Obaishi, H., Wada, M., Takahashi, K., Satoh, K., Hirao, K., Nishioka, H., Hata, Y., Mizoguchi, A. and Takai, Y. (1998) Neurabin-II/Spinophilin, an actin filament-binding protein with one PDZ domain localized at cadherin-based cell–cell adhesion sites. *J. Biol. Chem.* **273**, 3470–3475.

Sparks, A.B., Hoffman, N.G., McConnell, S.J., Fowlkes, D.M. and Kay, B.K. (1996) Cloning of ligand targets: systematic isolation of SH3 domain-containing proteins. *Nat. Biotech.* **14**, 741–744.

Suh, K.S., Ting, Y.T. and Burr, J.G. (1998) An avian cDNA encoding a tyrosine-phosphorylated protein with PDZ, coiled-coil, and SAM domains. *Gene* **219**, 111–123.

Tsutsui, K., Maeda, Y., Tsutsui, K., Seki, S. and Tokunaga, A. (1997) cDNA cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. *Biochem. Biophys. Res. Comm.* **236**, 178–183.

Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Mammalian ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205–214.

Wechsler-Reya, R., Elliott, K., Herlyn, M. and Prendergast, G.C. (1997a) The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. *Canc. Res.* **57**, 3258–3263.

Wechsler-Reya, R., Elliott, K. and Prendergast, G.C. (1998) A role for the putative tumor suppressor Bin1 in Muscle cell differentiation. *Mol. Cell. Biol.* **18**, 566–575.

Wechsler-Reya, R., Sakamuro, D., Zhang, J., Duhadaway, J. and Prendergast, G.C. (1997b) Structural analysis of the

human BIN1 gene: evidence for tissue-specific transcriptional regulation and alternate RNA splicing. *J. Biol. Chem.* **272**, 31453–31458.

Wigge, P., Kohler, K., Vallis, Y., Doyle, C.A., Owen, D., Hunt, S.P. and McMahon, H.T. (1997a) Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis. *Mol. Biol. Cell* **8**, 2003–2015.

Wigge, P., Vallis, Y. and McMahon, H.T. (1997b) Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr. Biol.* **7**, 554–560.

Oncogene (1999) 18, 3564–3573

http://www.stockton-press.co.uk/onc

Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms

Katherine Elliott[1], Daitoku Sakamuro[1], Amithaba Basu[1], Wei Du[1], William Wunner[1], Peter Staller[2], Stefan Gaubatz[2], Hong Zhang[3], Edward Prochownik[3], Martin Eilers[2] and George C Prendergast*,[1]

[1]*The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA;* [2]*Institute for Molecular Biology and Tumour Research (IMT), Universitaet Marburg, Emil-Mankopffstrasse 2, 35033 Marburg, Germany;* [3]*Section of Hematology/Oncology, Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15213, USA*

The tumor suppressor Bin1 was identified through its interaction with the N-terminal region of Myc which harbors its transcriptional activation domain. Here we show that Bin1 and Myc physically and functionally associate in cells and that Bin1 inhibits cell proliferation through both Myc-dependent and Myc-independent mechanisms. Bin1 specifically inhibited transactivation by Myc as assayed from artificial promoters or from the Myc target genes ornithine decarboxylase (ODC) and α prothymosin (pT). Inhibition of ODC but not pT required the presence of the Myc binding domain (MBD) of Bin1 suggesting two mechanisms of action. Consistent with this possibility, a non-MBD region of Bin1 was sufficient to recruit a repression function to DNA that was unrelated to histone deacetylase. Regions outside the MBD required for growth inhibition were mapped in Ras cotransformation or HepG2 hepatoma cell growth assays. Bin1 required the N-terminal BAR domain to suppress focus formation by Myc whereas the C-terminal U1 and SH3 domains were required to inhibit adenovirus E1A or mutant p53, respectively. All three domains contributed to Bin1 suppression of tumor cell growth but BAR-C was most crucial. These findings supported functional interaction between Myc and Bin1 in cells and indicated that Bin1 could inhibit malignant cell growth through multiple mechanisms.

Keywords: c-Myc; transformation; tumor supressor; transcription

Introduction

Myc is a central regulator of cell proliferation and apoptosis that is frequently activated in human malignancy (reviewed in Henriksson and Lüscher, 1996; Prendergast, 1997; Facchini and Penn, 1998). In normal cells induced to divide, the levels of Myc increase and remain elevated, indicating it is required throughout the cell cycle for proliferation. Deregulated Myc expression is sufficient to drive quiescent cells into S phase to prevent cell cycle exit. Conversely, suppression of Myc blocks mitogenic signals and facilitates terminal differentiation. Myc can also induce apoptosis, a feature manifested in normal cells when its expression is enforced and uncoupled from the orchestration of other cell cycle regulatory events. Myc is thought to act in the guise of a transcription factor, but the exact mechanisms underlying its oncogenic and apoptotic properties remain obscure.

We previously identified a cellular polypeptide, Bin1, which interacts with the putative transcriptional activation domain of Myc (Sakamuro *et al.*, 1996). The interaction depends upon the integrity of the so-called Myc boxes, two evolutionarily conserved segments which are necessary for both cell transformation and apoptosis. Although its adaptor functions appear to be complex, several observations support the hypothesis that Bin1 is a tumor suppressor that controls cell cycle transit and proliferation. First, Bin1 inhibits cell transformation by Myc or adenovirus E1A (Sakamuro *et al.*, 1996). Second, Bin1 is related to RVS167, a negative regulator of the cell cycle in yeast (Bauer *et al.*, 1993). Third, although widely expressed in normal cells, Bin1 is poorly expressed or undetectable in ~50% of carcinoma cell lines and primary breast carcinomas examined (Sakamuro *et al.*, 1996). Fourth, deficits in expression are functionally significant, because Bin1 can inhibit the growth of tumor cells which lack endogenous expression (Sakamuro *et al.*, 1996). Fifth, similar to several other important tumor suppressors, Bin1 promotes differentiation in the myogenic pathway and its inhibition suppresses differentiation (Wechsler-Reya *et al.*, 1998). Finally, the human Bin1 gene has been mapped to chromosome 2q14 (Negorev *et al.*, 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.*, 1996), and recent investigations suggest that loss of Bin1 function may contribute to prostate tumor progression (unpublished observations). Evidence from genetic, *in vitro* biochemical association, and co-localization experiments supports interaction between Bin1 and Myc (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a) but *in vivo* physical association and functional interaction had not been documented. In addition, Bin1 was shown to inhibit growth by adenovirus E1A as well as Myc, but whether this reflected similar or different functions was undetermined. In this study, we show that Bin1 physically associates with Myc in cells and inhibits its transcriptional properties and that Bin1 can inhibit malignant cell growth through Myc-independent as well as Myc-dependent mechanisms.

*Correspondence: GC Prendergast
The first two authors contributed equally to this study
Received 29 October 1998; revised 14 January 1999; accepted 14 January 1999

These findings support a role for Bin1 in governing the oncogenic properties of Myc but indicate that Bin1 also has additional roles in cell growth regulation.

Results

Physical and functional association of Myc and Bin1 in cells

Coimmunoprecipitation and transcription activation experiments were performed to examine the ability of Myc and Bin1 to functionally associate in cells. Association of Myc and Bin1 was observed to coimmunoprecipitation from baculovirus-infected Sf9 cells and untransfected C2C12 myoblasts, where Bin1 function has been examined (Wechsler-Reya *et al.*, 1998), using NP40 buffer conditions previously shown to support interaction of Myc and Bin1 *in vitro* (150 mM NaCl and 0.1% NP40). Bin1 was extracted more readily than Myc by NP40 lysis buffer from Sf9 cells infected with recombinant c-Myc and Bin1 baculoviruses, consistent with the fact that efficient extraction of Myc requires harsher conditions (RIPA buffer and sonication (Hann *et al.*, 1983). However, the Myc complexes extracted under these conditions contained Bin1 as indicated by coimmunoprecipitation with Myc antibody (Figure 1a). Association was specific because co-expression of the negative control proteins RhoB or yeast ADA3 with Bin1 did not result in Bin1 precipitation (data not shown). Bin1 antibodies capable to recognizing native Bin1 protein bind to epitopes in the Myc binding domain (MBD) (Wechsler-Reya *et al.*, 1997a) so the reverse immunoprecipitation experiment was intractable. Experiments using epitope-tagged Bin1 species were inconclusive, because tags at either the C- or N-terminus of Bin1 were not recognized unless denaturing conditions were used (i.e. RIPA buffer) that did not preserve Myc interaction *in vitro* (Sakamuro *et al.*, 1996; data not shown). However, Myc-Bin1 association was similarly observed in C2C12 cells. Myc and Bin1 are each expressed in proliferating C2C12 cells with Bin1 in stochiometric excess (Wechsler-Reya *et al.*, 1998). When C2C12 is induced to differentiate (Blau *et al.*, 1985), Bin1 is upregulated while Myc is downregulated to undetectable levels (Wechsler-Reya *et al.*, 1998), providing a useful negative control for association. As before, Myc was extracted inefficiently by NP40 buffer but Bin1 was detected in Myc complexes that were immunoprecipitated by Myc antibody (Figure 1b). The presence of Bin1 in these complexes was not due to antibody artifact or another nonspecific cause, because Bin1 was not detected in similar immunoprecipitates prepared from differentiated cell extracts.

To determine whether Bin1 association affected the transcriptional properties of Myc, transient activation assays were performed using a variety of promoters documented to be physiologically activated by c-Myc. The experiments employed luciferase (luc) reporter genes driven by artificial Myc-responsive promoters containing either multimerized DNA binding sites upstream of a minimal viral promoter or by the 5′ regions of the Myc target genes ornithine decarboxylase (ODC) and α-prothymosin (pT) (Bello-Fernandez *et al.*, 1993; Eilers *et al.*, 1991). The two artificial reporter genes were p3XMycE1b-luc (Gupta *et al.*, 1993) and Gal_5-E1b-luc, which included either three Myc-binding sites or five yeast GAL4 binding sites upstream of the adenovirus E1b minimal promoter. The latter reporter was used where activation was driven by chimeric molecules containing Bin1 or the Myc N-terminal transactivation domain (aa 1–262) fused to the DNA binding domain of the yeast transcription factor GAL4 (Kato *et al.*, 1990). The ODC and target gene reporters were ODCΔluc and PrT-luc (Bello-Fernandez *et al.*, 1993; Desbarats *et al.*, 1996; Packham and Cleveland, 1997). Cells were transfected with reporter plasmids and vectors for c-Myc or GAL4-Myc, Bin1, or the MBD deletion mutant Bin1ΔMBD (Sakamuro *et al.*, 1996). Max was included in pT experiments for optimal activation of this gene as documented (Desbarats *et al.*, 1996). Western or Northern analyses confirmed exogenous gene expression in transiently transfected cells (data not shown). ODC activation experiments included as a positive control for N-terminal interaction and inhibition of Myc activation the retinoblastoma (Rb)-

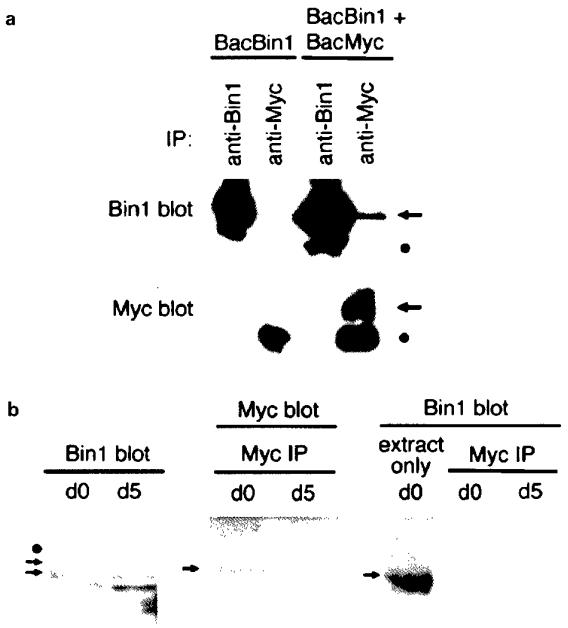


Figure 1 Biochemical association of Bin1 and Myc in cells. (**a**) Association in Sf9 cells. Extracts from 2×10^6 cells infected with the recombinant baculoviruses indicated were prepared and subjected to IP/Western analysis as described in the text and the Materials and methods. Dots indicate the position of coprecipitating antibodies recognized by anti-mouse or anti-rabbit secondary antibodies used to develop the blots, by a chemiluminescence technique. (**b**) Association in naive C2C12 myoblasts. Extracts from growing (d0) or differentiated (d5) C2C12 cells were prepared and subjected to Western or IP/Western analyses as described in the text and the Materials and methods. The left panel is a Western blot of an SDS gel loaded with 50 μg extract from d0 or d5 cells, demonstrating constitutive Bin1 expression and the appearance of a larger alternately spliced species in differentiated cells (Wechsler-Reya *et al.*, 1998).The dot indicates a nonspecific band. The middle panel is a Western blot of nonreducing SDS gel loaded with a Myc immunoprecipitate (sc-42) generated from 1.5 mg of d0 or d5 extracts and probed with a second anti-Myc antibody (9E10). The right panel is a Western blot of a nonreducing SDS gel loaded with 50 μg of d0 extract alone or a Myc immunoprecipitate (sc-42) from 1.5 mg d0 or d5 extracts and probed with anti-Bin1 99D

related protein p107 (Beijersbergen *et al*., 1994; Gu *et al*., 1994).

Bin1 selectively inhibited Myc activation on all the Myc reporter promoters tested (Figure 2). In NIH3T3 cells, Myc activated p3XMycE1b-luc ~2.5-fold, similar to the level observed by others (Kretzner *et al*., 1992), and titration of Bin1 into the assay reversed the effect of Myc (Figure 2a). Similarly, Myc activated the ODC promoter ~2.3-fold, also as documented previously (Packham and Cleveland, 1997), and Bin1 reversed this effect as potently as p107 (Figure 2b). Deletion of the Myc-binding domain (MBD) from Bin1 relieved its ability to inhibit ODC in both HeLa and NIH3T3 cells (Figure 2c). The inability of Bin1ΔMBD to suppress Myc was not due to polypeptide instability nor to general loss of function, because Bin1ΔMBD accumulated similarly to wild-type Bin1 in transfected COS cells and because Bin1ΔMBD could inhibit E1A transformation (see below). A more robust activation of pT by Myc-Max was also inhibited by Bin1 ~3-fold (Figure 2d). Bin1ΔMBD also inhibited Myc activation of pT indicating the effect on this gene was MBD-independent. However, inhibition was specific because Bin1 did not affect activation by VP16. Western analysis confirmed Myc and Max accumulation in transiently transfected cells, ruling out the trivial possibility that Bin1 acted by inhibiting the exogenous Myc or Max expression (data not shown). The specificity of the effect of Bin1 for the Myc N-terminus was investigated using GAL4-Myc or a second GAL4 chimera which included instead the activation domain from the nonspecific but broadly active herpes virus activator VP16 (GAL4-VP16). For these experiments, we examined activation of a pT reporter (GAL4mE-prT-luc) that was identical to the prT-luc reporter used above except that the two Myc

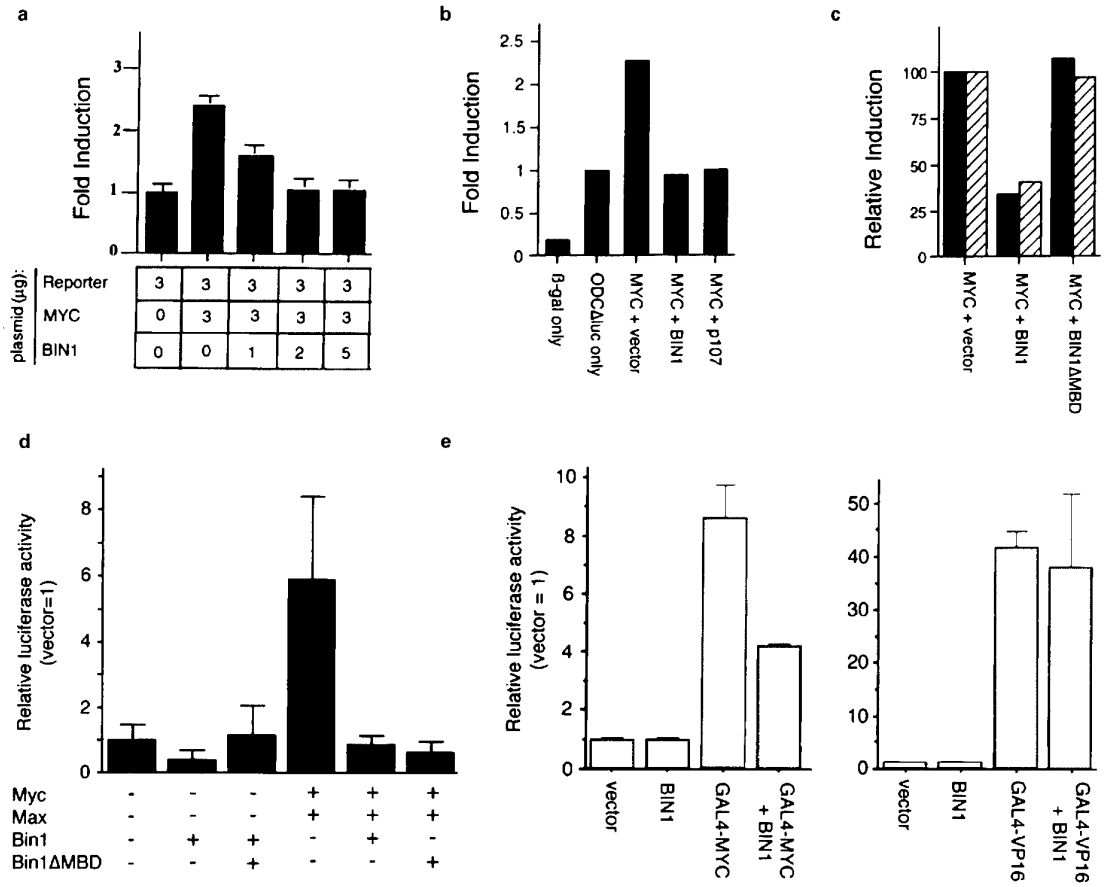


Figure 2 Bin1 specifically inhibits gene activation by Myc. (**a**) Inhibition of a Myc-responsive artificial promoter. NIH3T3 cells were transfected with the plasmids indicated by standard calcium phosphate method and processed for normalized luciferase activity as described (Zhang and Prochownik, 1997). The data represent the results of three trials each performed in duplicate. (**b**) Inhibition of ODC activation. NIH3T3 cells seeded into six well dishes were transfected with 1.5 μg of the ODC reporter ODCΔluc, 3 μg of the human c-Myc vector LTR Hm, 1.5 μg CMV Bin1 or CMV p107, and 0.5 μg CMV-βgal (to normalize for transfection efficiency). pcDNA3 was added to equalize the amount of plasmid in each transfection. Two days later cell extracts were prepared and processed for normalized reporter activity. The graph depicts relative luciferase activity based on reporter only (set at 100%); the absolute values ranged from 10^3-10^4 light units. The results represent the average of two trials each performed in duplicate. (**c**) MBD is required for ODC inhibition. NIH3T3 or HeLa cells were transfected with 0.5 μg ODCΔluc and 2 μg LTR-Hm plus 3.25 μg vector, CMV-Bin1, or CMV-Bin1ΔMBD. Cell extracts were prepared and processed as above. The results represent the average of two trials performed in duplicate. Relative luciferase activity is depicted as the proportion of reporter plus LTR Hm; the absolute values ranged from 10^3-10^4 light units. (**d**) Inhibition of pT activation. HeLa cells were transfected with PrT-luc, a β-galactosidase normalization plasmid, and the vectors indicated as described (Desbarats *et al*., 1996). Where indicated Bin1 or control plasmids were included in a 1:1 w/w ratio with Myc. Relative luciferase activity is depicted as above; the absolute values ranged from 10^4-10^6 light units. (**e**) Bin1 inhibits GAL4-Myc but not GAL-VP16. HeLa cells were transfected with GAL4mE-PrT-luc and the genes indicated as above and processed for relative luciferase activity

binding sites in the gene were replaced with GAL4 binding sites (Desbarats *et al.*, 1996). Bin1 inhibited activation of pT by GAL-Myc but not by GAL4-VP16 (Figure 2e). Similar results were obtained with GAL4-E1b-luc (data not shown). Taken together, the results of the immunoprecipitation and transcription experiments argued that Bin1 physically and functionally interacted with Myc in cells.

Bin1 can recruit a transcriptional repression function to DNA

Bin1 does not harbor motifs characteristic of transcription adaptor proteins, so one interpretation of the above results was that Bin1 acted via a passive mechanism, for example, by occluding contacts with as yet unidentified coactivators or with the TATA-binding protein (TBP), which has been reported to interact with Myc (Hateboer *et al.*, 1993). Alternately, Bin1 may act through an active repressive mechanism, perhaps by recruiting a corepressor to the promoter similar to the Mad-binding protein mSin3 (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). To assess the latter hypothesis, we tested the effects of Bin1 on basal transcription of a promoter to which it was tethered in a Myc-independent manner. This was achieved by fusing Bin1 in frame to the DNA binding domain of GAL4 to generate GAL4-Bin1. A second GAL4 chimera that lacked the MBD was constructed (GAL4-Bin1ΔMBD) to eliminate MBD-dependent interactions with Myc, Myc-binding coactivators yet to be identified, or possibly TBP (Hateboer *et al.*, 1993), all of which might mask repressive effects or make their interpretation more difficult. HeLa cells were transfected with the artificial promoter-reporter gene GAL_5-E1b-luc and equivalent amounts of expression vectors for unfused GAL4 DNA binding domain (GAL0), GAL4-Bin1, or GAL4-Bin1ΔMBD and cell lysates were processed for luciferase activity as before. GAL4-Bin1 was only slightly inhibitory but GAL4-Bin1ΔMBD repressed basal transcription ~2.5-fold relative to unfused GAL0 (Figure 3a). GAL4-Bin1ΔMBD had little effect on the activity of luciferase reporters lacking GAL4 sites (data not shown), indicating that this effect was dependent on DNA binding. To determine if repression reflected recruitment of a Bin1-binding factor, we added vector, wild-type (untethered) Bin1, or Bin1ΔMBD to the cotransfection. If the activity was intrinsic, cotransfection of Bin1 would not affect repression, whereas if repression was due to recruitment of a *trans*-acting factor then untethered Bin1 would be predicted to titer the repressive effect. Consistent with the latter case, both Bin1 and Bin1ΔMBD relieved repression by GAL4-Bin1ΔMBD (Figure 3b). The greater relief provided in Bin1ΔBD suggested that a region outside of the MBD might recruit a repression function. Experiments in which trichostatin A was added did not relieve the repressive effect of GAL4-Bin1ΔMBD suggested that a region outside of the MBD might recruit a repression function. Experiments in which trichostatin A was added did not relieve the repressive effect of GAL4-Bin1ΔMBD (data not shown), suggesting that this function was not a histone deacetylase and that Bin1 acts differently than mSin3 (Facchini and Penn, 1998). Nevertheless, the results suggested that Bin1 may actively inhibit Myc activation by recruiting a repression function.

Expression and localization of Bin1 deletion mutants

To identify non-MBD regions that are important for Bin1 activity a set of deletion mutants was constructed

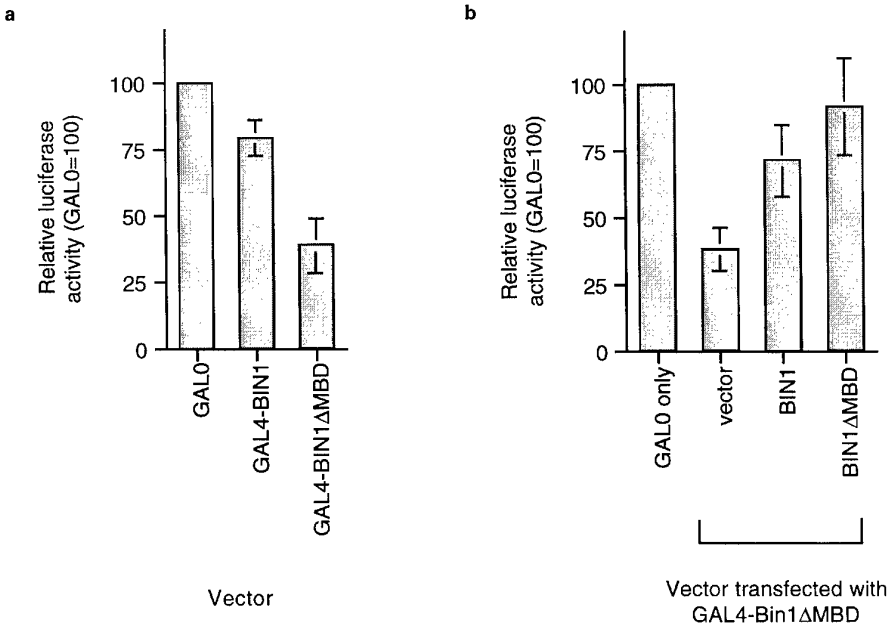
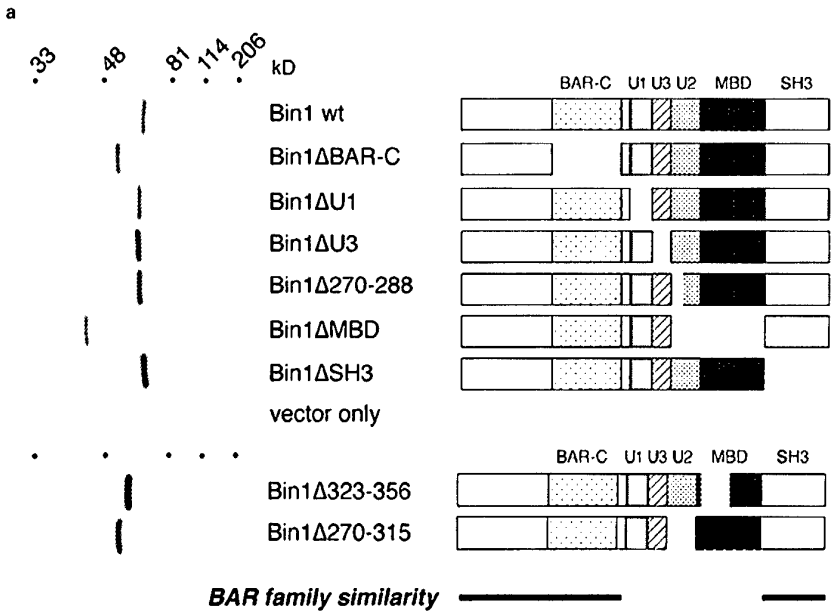


Figure 3 Bin1 recruits a repression function when tethered to a promoter. (**a**) Intrinsic repressive quality of Bin1. HeLa cells were transfected with 2 μg GAL4-SV40-luc reporter and 4 μg of the indicated GAL4 chimeric gene and normalized luciferase activity was determined 2 days later. The data represent the results of at least four trials each performed in duplicate. (**b**) The repressive activity of BIN can be titered. Cells were transfected with 2 μg GAL4-SV40-luc reporter, 4 μg of GAL4-Bin1ΔMBD, 4 μg CMV vector, Bin1, or Bin1ΔMBD plasmids and normalized luciferase activity was determined 2 days later. The data represent the results of four trials each performed in duplicate

(Figure 4). BAR-C and SH3 encompass regions of Bin1 that are related to the neuron-specific protein amphiphysin and to the yeast cell cycle regulator RVS167 (the BAR nomenclature reflects the Bin1/



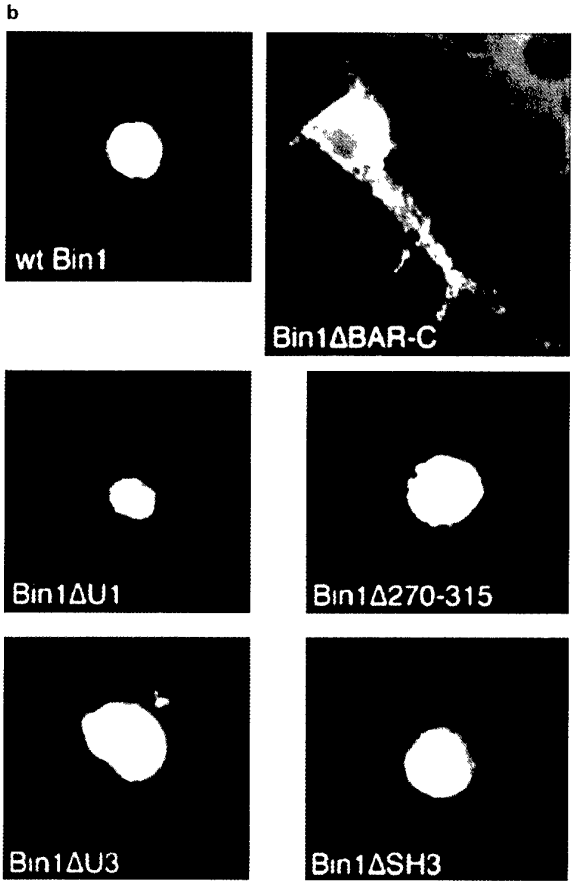


Figure 4 Structure, expression, and localization of Bin1 deletion mutants. (**a**) Expression of Bin1 mutants. COS cells were transfected with the expression vectors indicated, metabolically labeled with ^{35}S-methionine/cysteine, and cell extracts were prepared and subjected to immunoprecipitation with Bin1 monoclonal antibodies. Immunoprecipitates were examined by SDS–PAGE and fluorography. The bars at the bottom of the cartoon denote regions that are structurally related among proteins of the BAR family, which includes Bin1, amphiphysin, and RVS167 (Sakamuro *et al*., 1996). (**b**) Localization of Bin1 mutants. 293T cells seeded on glass cover slips were transiently transfected with the expression vectors and processed for indirect immunofluorescence with Bin1 monoclonal antibody 99D as described in the Materials and methods

amphiphysin/RVS167 homology in this region; BAR-C represents the C-terminal half of the BAR domain (Figure 4a). The SH3 domain located at the C-terminus is dispensable for interaction with Myc (Sakamuro *et al.*, 1996). The central region is not conserved in amphiphysin or RVS167 and is unique to Bin1. This region includes the so-called unique-1 (U1) region encoded in the human gene by exon 9; the alternately spliced and strongly positively charged unique-3 (U3) region encoded by exon 10; the unique-2 (U2) region encoded by exon 11 which harbors two copies of the SH3 binding motif PXXP; and the MBD (Wechsler-Reya *et al.*, 1997b). The MBD as initially defined encompassed aa 270–389. Deletions of three subsections of this segment were generated for this study, aa 270–288, aa 270–315 (comprising the newly defined U2 region) and aa 323–356 (N-terminal half of the MBD). Expression of the mutant polypeptides was confirmed by immunoprecipitation from COS cell extracts. Cells were transfected with vectors for each mutant, metabolically labeled with 35S-methionine, and extracts were prepared and processed for immunoprecipitation with a mixture of Bin1 monoclonal antibodies (Wechsler-Reya *et al.*, 1997a). The apparent and predicted MWs of the mutants did not coincide in each case because of the presence of a determinant for aberrant gel mobility that maps to the MBD region (Sakamuro *et al.*, 1996). Each mutant was observed to accumulate as efficiently as full-length Bin1 (Figure 4a). The cell localization of several mutants was examined by indirect immunofluorescence in transiently transfected 293T cells (Figure 4b). The presence of an SV40 replication origin on the expression vectors made it possible to distinguish cells expressing exogenous proteins by using a higher dilution of Bin1 monoclonal antibody than needed to detect endogenous expression (1 : 100 instead of 1 : 5 dilution). Consistent with previous results (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a), wild-type Bin1 localized exclusively to the nucleus, as did Bin1 mutants lacking the U1, U2, U3 and SH3 regions (Bin1ΔU1 was also preferentially excluded from the nucleolus). U3 contains a nuclear localization motif but its dispensability for nuclear localization was consistent with recent findings in which alternate splicing of the exon encoding U3 after myoblast differentiation is correlated with the appearance of cytosolic Bin1 species (Wechsler-Reya *et al.*, 1998; Wechsler-Reya *et al.*, 1997b). Instead, BAR-C contained a critical nuclear localization signal, because both nuclear and cytosolic staining was detected in cells transfected with Bin1ΔBAR-C. We concluded that BAR-C sequences between aa 125–207 included signal(s) for nuclear localization and/or retention.

Bin1 inhibits malignant cell transformation by multiple mechanisms

Using the Ras cooperation assay performed in primary rat embryo fibroblasts (REFs) (Land *et al.*, 1983; Ruley, 1983), we previously showed that Bin1 inhibits malignant transformation by c-Myc in a MBD-dependent manner (Sakamuro *et al.*, 1996). To define other regions required, REFs were transfected with expression vectors for Myc, oncogenic Ras, and Bin1 or Bin1 deletion mutants, and transformed cell foci were scored 2 weeks later (Figure 5). Consistent with previous results (Sakamuro *et al.*, 1996), wild-type Bin1 suppressed focus formation by Myc ~sixfold relative to the empty vector control. Most deletion mutants inhibited focus formation as efficiently as wild-type Bin1, suggesting modularity in the structural organization of this polypeptide. Only BAR-C or the MBD segment aa 323–356 were required, identifying BAR-C determinants as crucial to inhibit Myc transformation along with the MBD. Since aa 270–315 (U2 region) was dispensable for inhibiting Myc transformation the critical part of the MBD therefore was confined to a 66 residue segment between aa 323–389. The inactivity of the MBD aa 323–356 or BAR-C deletion mutants was not due to protein instability, because each polypeptide accumulated similar to wt Bin1 in COS cells (Figure 4a), nor to misfolding, because each polypeptide efficiently suppressed transformation by E1A or mutant p53. Bin1ΔBAR-C localized to the nucleus and cytoplasm (Figure 4b) but its ability to suppress E1A and mutant p53, which act in the nucleus, also argued against mislocalization as the cause for loss of activity against Myc. We previously showed that Bin1 inhibited transformation by adenovirus E1A but not SV40 large T antigen (Sakamuro *et al.*, 1996), and in this study we show that Bin1 also inhibited transformation by dominant inhibitory mutant p53. Bin1 suppressed transformation by E1A or mutant p53 ~threefold (Figure 6); the inhibitory effects of each could be titered as was the case with Myc (Sakamuro *et al.*, 1996) by altering the ratio of Bin1 to E1A or mutant p53 in the assay (data not shown). U1 was crucial to inhibit E1A and U1 and SH3 were both crucial to inhibit mutant p53 (Figure 6). U3, BAR-C, and MBD were each dispensable to inhibit either oncoprotein. As before, neither protein instability nor misfolding was responsible for the loss of activity of either mutant since each accumulated in COS and each could suppress Myc transformation (Figures 4a and 5).

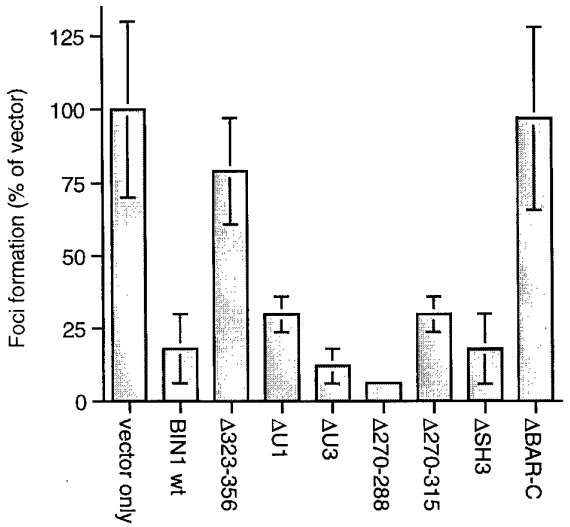


Figure 5 BAR-C is required to inhibit Myc transformation. REFs were transfected with 5 μg each oncogenic Ras and deregulated human c-Myc plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12–14 days later. The data depict the percentage of Myc+Ras foci formed in the presence of empty vector

Northern analyses of RNA isolated from pools of foci derived from Myc+Ras, E1A+Ras, or mutant p53+Ras transfections showed that, as predicted, mutant Bin1 messages accumulated in transformed cells if the mutant was biologically inactive. For example, Bin1ΔBAR-C message only accumulated in Myc+Ras foci whereas Bin1ΔU1 message only accumulated in E1A+Ras or mutant p53+Ras foci (data not shown). Thus, the domains required to inhibit E1A and mutant p53 were distinct from those required to block Myc. The importance of the BAR-C domain to the inhibitory activity of Bin1 was confirmed in HepG2 cells (Figure 7). Deletion of other domains only partly relieved HepG2 growth consistent with the likelihood that multiple growth mechanisms were deregulated in these tumor cells (data not shown). Notably, MBD deletion also only slightly relieved suppression, underscoring the importance of MBD-independent mechanisms for some types of growth inhibition by Bin1. Since neither E1A nor mutant p53 require endogenous Myc to transform cells, the differences in domain dependence argued that Bin1 could regulate malignant cell proliferation through Myc-independent as well as Myc-dependent mechanisms.

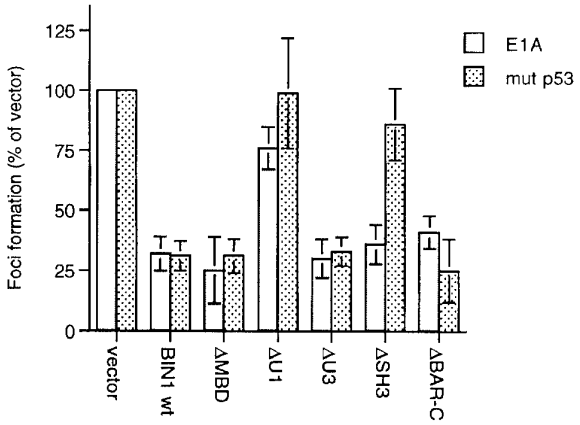


Figure 6 U1 is required to inhibit E1A transformation and U1 and SH3 are each required to suppress mutant p53 transformation. REFs were transfected with 5 μg each oncogenic Ras and adenovirus E1A or dominant inhibitory p53 mutant plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12–16 days later. The data depict the percentage of E1A+Ras or mutant p53+Ras foci formed in the presence of empty vector

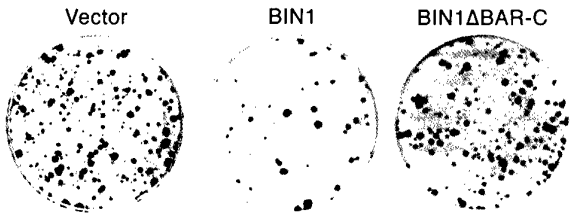


Figure 7 BAR-C is crucial to suppress tumor cell growth. HepG2 cells were transfected with 2 μg of neomycin (neo^r)-resistance gene marked vectors. G418-resistant cell colonies were scored ~3 weeks later by methanol fixation and cystal violet staining. A representative assay is shown from multiple experiments performed in triplicate

Discussion

This study supports the assertion that Myc and Bin1 physically and functionally associate in cells, and it showed that Bin1 can inhibit malignant cell proliferation by both Myc-dependent and Myc-independent mechanisms (Figure 8). Myc-Bin1 complexes were detected by coimmunoprecipitation from recombinant baculovirus-infected Sf9 cells or from naive C2C12 cells. The fact that Myc-Bin1 complexes could be identified in growing C2C12 cells suggested that association is not inhibitory *per se* but may be subjected to posttranslational regulation. This possibility would be consistent with demonstrations that Bin1 is phosphorylated and associated *in vivo* with other proteins in addition to Myc (Wechsler-Reya *et al*., 1997a). The ability of Bin1 to specifically inhibit Myc function as measured by activation of artificial and natural target genes supported *in vivo* association. Activation by Myc/Max or by GAL4-Myc chimeras containing the Myc transactivation domain, but not by GAL4-VP16, was susceptible to Bin1 inhibition. VP16 is a complex activator that can act through a variety of adaptors, so the fact that VP16 was not inhibited by Bin1 indicated that its activity was specific and not due to nonselective suppression of transcriptional activation. ODC and pT are two paradigm target genes for Myc and the ability of Bin1 to inhibit each supported the notion of functional interaction. Whether Bin1 has

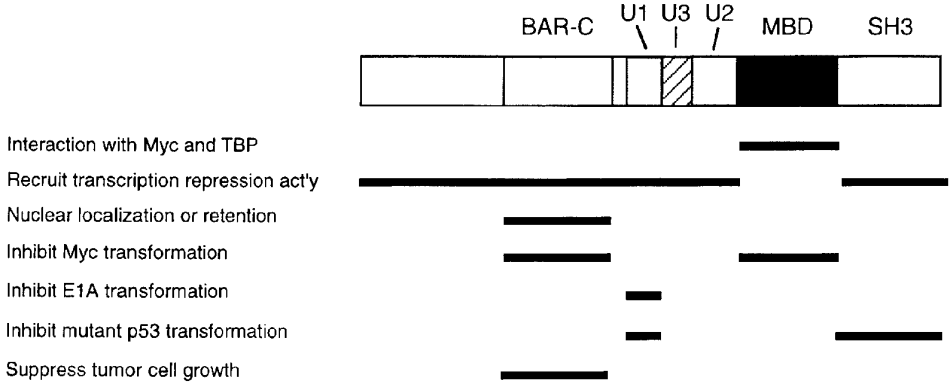


Figure 8 Summary of Bin1 functions. Myc interaction data is from Sakamuro *et al*. (1996)

a physiological role in transcription bears further analysis. However, in support of this possibility we showed that Bin1 could recruit a potential repression function to DNA via an MBD-independent interaction. In addition, Myc has been reported to interact *in vitro* with TATA-binding protein (Hateboer *et al.*, 1993) and we have observed that the Bin1 MBD can avidly bind TBP *in vitro* (D Sakamuro and GC Prendergast, unpublished observations). Although the consequences of Myc-TBP interaction have not been established *in vivo* the ability of Bin1 to bind TBP conceivably represents a second mechanism through which Bin1 could disrupt Myc activation. As considered above, it is possible that the inhibitory effects of Bin1 on Myc activation are passive and an epiphenomenon of steric occulusion of coactivators which are yet to be identified. Recent results indicated that Bin1 is necessary for Myc-mediated apoptosis (D Sakamuro, J Duhadaway and GC Prendergast, unpublished observations) would provide a biological foundation to assess the physiological significance of the putative transcriptional properties of Bin1 documented in this study.

The N-terminal BAR-C region of Bin1 was required to inhibit Myc transformation. BAR-C is a charged region of 84 aa predicted to be both α helical and involved in coiled-coil interactions (Lupas, 1996). A key functional role for this region is supported by the fact that it contains the most highly conserved sequences in Bin1 in evolution. Given the requirement for MBD and BAR-C to suppress Myc transformation one might have expected both regions to be important for the inhibitory effects of Bin1 in HepG2, which overexpresses Myc. However, if Myc-independent growth pathways deregulated in HepG2 are dominant or co-dominant with Myc-dependent pathways then this would not be expected to be the case. BAR-C included a signal(s) for nuclear localization or retention, while NLS-like sequences in U3 (Sakamuro *et al.*, 1996) have been shown here and elsewhere (Wechsler-Reya *et al.*, 1998) to be dispensable. The results of this study mapped the MBD within a 61 residue segment between aa 315–376 immediately upstream of the SH3 domain. Interestingly, this region of Bin1 is encoded by two exons and the more 5′ exon has been found to be alternately spliced in cells (Wechsler-Reya *et al.*, 1997b). The aa 323–356 deletion which relieved Myc suppression activity closely overlaps the sequences encoded by this exon. Thus, one alternately spliced Bin1 species in cells probably lacks Myc binding capacity and functions independently of Myc, a likelihood that is consistent with Myc-independent growth inhibitory properties of Bin1 identified in this study. Alternative splice forms of Bin1 that are neuron-specific, termed amphiphysin-like isoform or amphiphysin II, have been implicated in endocytosis (Wigge and McMahon, 1998). However, we do not believe endocytosis is relevant to the Myc-independent growth inhibition mechanisms identified here, because non-neuronal splice forms lack determinants required for interaction with endocytosis systems (Ramjaun and McPherson, 1998) and because the inclusion of neuron-specific exons in Bin1 eliminates its growth inhibitory activity (unpublished observations).

The C-terminal U1 and SH3 regions were required to inhibit transformation by E1A or p53 but not by Myc. U1 is contained on a single exon which encodes 28 aa (Wechsler-Reya *et al.*, 1997b). E1A transforms cells by displacing E2F from Rb (Dyson and Harlow, 1992) so U1 either impedes this process somehow or acts downstream to interfere with E2F effectors. Consistent with a link between U1 and the Rb/E2F system, U1 deletion also blocks transformation by the human papilloma virus E7 protein (data not shown), which acts similarly to E1A by interfering with Rb/E2F interaction (Phelps *et al.*, 1988). The requirement of U1 to inhibit mutant p53 is consistent with evidence that cell transformation by mutant p53 also depends on interference with Rb/E2F interactions (Hansen *et al.*, 1995). The SH3 domain of Bin1 was also necessary to inhibit transformation by mutant p53. To our knowledge Bin1 and Abl are the only two SH3-containing proteins localized to the nucleus, and recently Abl has been shown to interact with Bin1 in an SH3-dependent manner (Kadlec and Pendergast, 1997; D Sakamuro and GC Prendergast, unpublished observations). This may be of consequence since Abl and p53 have been reported to interact in cells (Yuan *et al.*, 1996), although the physiological significance of this interaction has not been established clearly. Direct interaction between the Bin1 SH3 and the PxxP motifs in the apoptosis effector domain of p53 (Sakamuro *et al.*, 1997) could be germane since PxxP motifs constitute SH3 ligands. Indeed, since this region also has been implicated in growth inhibition (Walker and Levine, 1996) and the transforming efficiency of mutant p53 rests upon more than simple inactivation of endogenous p53 (Dittmer *et al.*, 1993; Hulboy and Lozano, 1994), it is conceivable that mutant p53 may promote transformation in a PxxP-dependent manner by sequestering a nuclear SH3-containing growth suppressor such as Bin1.

Materials and methods

Plasmid constructions

The following plasmids have been described. CMV-Bin1 and CMV-Bin1ΔMBD encode full-length Bin1 or an MBD deletion mutant, respectively (Sakamuro *et al.*, 1996). LTR Hm contains a Moloney retroviral long terminal repeat-driven normal human c-*myc* gene (Kelekar and Cole, 1986); pSVLneo-C-myc is an SV40 early region-driven c-Myc vector used in Figure 2a that has been described (Zhang and Prochownik, 1997); p1A/neo contains the 5′ end of the adenovirus type 5 genome including the E1A region (Maruyama *et al.*, 1987); LTR p53ts encodes a temperature-sensitive dominant inhibitory mutant of murine p53 (Michalovitz *et al.*, 1990); and pT22 contains an activated H-ras gene (Land *et al.*, 1983). CMV-p107 contains a full-length human p107 cDNA (Zhu *et al.*, 1993) in the cytomegalovirus enhancer/promoter-containing vector pcDNA3 (Invitrogen). P3XMyc-Elb-luc is an artificial reporter gene containing multimerized CACGTG Myc E box sequences upstream of the minimal adenovirus E1b promoter (Gupta *et al.*, 1993). GAL4-E1b-luc and GAL4-SV40-luc are GAL4 reporters which contain multimerized GAL4 sites upstream of the minimal E1b or SV40 early promoters (gifts of F Rauscher III). The ODC luciferase reporter ODCΔluc and the α-prothymosin luciferase reporters PrT-luc and GAL4mE-PrT-luc have been described (Desbarats *et al.*, 1996; Packham and Cleveland, 1997). The BacBin baculovirus was prepared by standard methods (O'Reilly *et al.*, 1992) using the baculovirus expression vector pVL1392 (Invitrogen) into which the full-

length Bin1 cDNA was subcloned. A murine c-Myc baculovirus (a gift of M Cole) was prepared similarly. GAL0 is the DNA binding domain of GAL4 (aa 1–143) and GAL4-Myc includes aa 1–262 of human Myc except the b/HLH/LZ region (Kato *et al*., 1990) which is necessary to bind Max (Prendergast *et al*., 1991). Bin1 deletion mutants and GAL4 fusion genes were subcloned for expression in pcDNA3 (the same vector as Bin1 and Bin1ΔMBD). Bin1ΔBAR-C was constructed by dropping an internal Afl III restriction fragment from CMV-Bin1, resulting in a deletion of aa 125–207 from the BAR domain (Sakamuro *et al*., 1996). The remaining mutants were generated by standard PCR methodology using the oligonucleotide primers 995′(Bam), 993′SH3(Xho) (Sakamuro *et al*., 1996) and others whose sequence is derived from the Bin1 cDNA sequence (GenBank accession number U68485). The integrity of PCR-generated fragments was verified by DNA sequencing. To conserve space, oligonucleotides and construction details are omitted but are available from GC Prendergast. Bin1ΔU1 lacks aa 224–248; Bin1ΔNLS, aa 251–269; Bin1ΔSH3, aa 384–451; the other mutants lack the residues indicated. GAL4-Bin1 fusions were generated in two steps by first subcloning the 143 aa DNA binding domain from GAL0 into pcDNA3 and then ligating in-frame full-length Bin1 or Bin1ΔMBD (lacking aa 270–383) cDNAs downstream.

Cell culture

COS, 293T, HeLa, and HepG2 cells from the ATCC were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Atlantic) and 40 U/ml penicillin and streptomycin (Fisher). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Gibco) and antibiotics (transfections were performed in media containing 10% fetal calf serum). C2C12 cells were carried in DMEM supplemented with 15% fetal bovine serum and antibiotics. Differentiation of C2C12 was induced by removing shifting cells at ~70% confluence to DMEM supplemented with 5% horse serum and antibiotics for 5 days, when myotube formation was maximal. Secondary passage rat embryo fibroblasts (REFs) were obtained from Whittaker Bioproducts and cultured and transfected as described (Prendergast *et al*., 1992). For transformation assays, secondary passage REFs seeded in 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method (Chen and Okayama, 1987) with 5 μg each of oncogenic Ras plus Myc, E1A, or mutant p53 expression plasmids and 10 μg of Bin1 plasmid or empty vector. Cells were fed and the next day passaged into one 15 cm dish (Myc transfections) or three 10 cm dishes (E1A or mutant p53 transfections). Foci were scored by methanol fixation and crystal violet staining 12–16 days later. Colony formation assays in HepG2 cells were performed by seeding ~3×10^5 cells in 6 cm dishes and transfecting the next day with 2 μg plasmid DNA using Lipofectamine (Gibco/BRL). Cells were passaged 48 h after transfection at a 1:10 ratio into 6 cm dishes containing media with ~0.6 mg/ml G418 and cell colonies were scored by crystal violet staining ~3 weeks later.

Immunoprecipitation

For insect cell experiments, ~10^7 Sf9 cells were infected with the recombinant baculoviruses indicated at an m.o.i. of approximately 10. Two days after infection, cells were harvested and ~2×10^6 cells for each IP were extracted in 0.5 ml 50 mM TrisCl pH 8/150 mM NaCl/0.1% NP40. Clarified lysates were subjected to immunoprecipitation by incubation 1.5 h at 4°C with 1 μg of anti-murine c-Myc antibody #6–213 (Upstate Biotechnology) or 100 μl hybridoma supernatant containing the Bin1 monoclonal antibody 99D (Wechsler-Reya *et al*., 1997a). Immune complexes were collected on Protein G-Sepharose (Pharmacia), washed four times with binding buffer, eluted by boiling in SDS gel loading buffer, and fractionated by SDS–PAGE. Gels were Western blotted by standard methods (Harlow and Lane, 1988) and probed with 1 μg/ml anti-Myc or a 1:50 dilution of 99D hybridoma supernatant. Blots were developed using a chemiluminescence kit (Pierce). For experiments in mouse cells, 5–10 dishes of growing or differentiated C2C12 cells were trypsinized, washed with excess growth media and then with 30 ml PBS each at 4°C. All subsequent steps were performed on ice or at 4°C. Cells were resuspended in hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 0.1 mM EDTA, and 1 mM PMSF, aprotinin, leupeptin, antipain), incubated 3–5 min, and pelleted. These swollen cells were resuspended in extraction buffer (20 mM HEPES, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 0.1% NP-40, and protease inhibitors) and lysed by 10 strokes with a B pestle homogenizer. Before immunoprecipitation, the extract was incubated 15 min and clarified by a 5 min microcentrifugation. The protein concentration in the extract was determined by Bradford assay and 1.5 mg was incubated overnight with 1 μg anti-c-myc #sc-42 (Santa Cruz Biotechnology). Immune complexes were collected on protein G-agarose, washed three times with extraction buffer, and fractionated by nonreducing SDS–PAGE. Gels were Western blotted and probed as indicated with a 1:50 dilution of 99D hybridoma supernatant or ~1 μg/ml anti-Myc antibody 9E10 (Evan *et al*., 1985). Blots were developed in these experiments with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase, using an colormetric staining reaction catalyzed by this enzyme. To confirm expression of Bin1 deletion mutants, COS cells were metabolically labeled for 2 h in DMEM lacking methionine and cysteine (Gibco) with 100 μCi/ml EXPRESS labeling reagent (NEN) and cell extracts were prepared with NP40 buffer containing leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and antipain (Harlow and Lane, 1988). Extracts were microcentrifuged for 15 min at 4°C before use. Extracts were precleared by a 1 h treatment with prebleed sera or normal mouse IgG and 20 μl or a 1:1 slurry of protein G-Sepharose beads at 4°C on a nutator (Pharmacia). A mixture of hybridoma supernatants (50 μl each) containing Bin1 monoclonal antibodies 99D, 99E, and 99I were used for immunoprecipitation (Wechsler-Reya *et al*., 1997a). After incubation 1 h at 4°C, immune complexes were collected on protein G-Sepharose, washed four times with NP40 buffer, eluted in SDS gel loading buffer, fractionated on 10% SDS-PA gels, and fluorographed.

Immunofluorescence

293T were seeded onto glass cover slips in six well dishes and transfected the next day with 5 μg of the Bin1 expression vectors indicated. Two days later, cells were fixed, lysed, and processed for Bin1 immunofluorescence as described previously (Prendergast and Ziff, 1991; Sakamuro *et al*., 1996; Wechsler-Reya *et al*., 1997a), except that a 1:100 instead of a 1:5 dilution of 99D was used to limit detection to cells overexpressing the gene products of interest. Cells were photographed on a Leica immunofluorescence microscope apparatus using Ektachrome film and slides were scanned and processed with Photoshop software.

Transactivation assays

Conditions for transient Myc activation assays were taken from the reports using the various reporter genes employed (Bello-Fernandez *et al*., 1993; Desbarats *et al*., 1996; Kato *et al*., 1990; Packham and Cleveland, 1997; Zhang and Prochownik, 1997). NIH3T3 or HeLa cells were transfected using standard calcium phosphate methods and promoter sequences and total plasmid DNA in each transfection was equalized with empty vectors as appropriate. Each DNA mixture included equivalent amounts of a β-galactosidase

vector to normalize for transfection efficiency. Two days after transfection, cell extracts were prepared and analysed for luciferase and β-galactosidase activity using commercial kits, following protocols provided by the vendor (Promega).

Acknowledgements
We thank Roberto Buccafusca for technical assistance, John Cleveland for the ODCΔluc reporter plasmid, Michael Cole for a murine c-Myc baculovirus, Chi Dang for GAL4-Myc plasmids, and Karl Munger for HPV E7 and control vectors. For discussion and critical comments, we thank Shelley Berger, Frank Rauscher III, and members of our laboratory. This work was supported by grants from the ACS and US Army Breast Cancer Research Program. Katherine Elliott was supported by an NIH Training Grant. George C Prendergast is a Pew Scholar in the Biomedical Sciences.

References

Ayer DE, Lawrence QA and Eisenman RN. (1995). *Cell*, **80,** 767–776.
Bauer F, Urdaci M, Aigle M and Crouzet M. (1993). *Mol. Cell. Biol.*, **13,** 5070–5084.
Beijersbergen RL, Hijmans EM, Zhu L and Bernards R. (1994). *EMBO J.*, **13,** 4080–4086.
Bello-Fernandez C, Packham G and Cleveland JL. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 7804–7808.
Blau HM, Pavlath GK, Hardeman EC, Chiu CP, Silberstein L, Webster SG, Miller SC and Webster C. (1985). *Science*, **230,** 758–766.
Chen C and Okayama H. (1987). *Mol. Cell Biol.*, **7,** 2745–2752.
Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB and Jensen RH. (1996). *Cancer Res.*, **56,** 3091–3102.
Desbarats L, Gaubatz S and Eilers M. (1996). *EMBO J.*, **10,** 447–460.
Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C and Levine AJ. (1993). *Nat. Genet.*, **4,** 42–46.
Dyson N and Harlow E. (1992). *Cancer Surv.*, **12,** 161–195.
Eilers M, Schirm S and Bishop M. (1991). *EMBO J.*, **10,** 133–141.
Evan GI, Lewis GK, Ramsay G and Bishop JM. (1985). *Mol. Cell Biol.*, **5,** 3610–3616.
Facchini LM and Penn LZ. (1998). *FASEB J.*, **12,** 633–651.
GU W, Bhatia K, Magrath IT, Dang CV and DallaFavera R. (1994). *Science*, **264,** 251–254.
Gupta S, Seth A and Davis RJ. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 3216–3220.
Hann SR, Abrams HD, Rohrschneider LR and Eisenman RN. (1983). *Cell*, **34,** 789–798.
Hansen R, Reddel R and Braithwaite A. (1995). *Oncogene*, **11,** 2535–2545.
Harlow E and Lane D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
Hateboer G, Timmers H, Rustgi AK, Billaud M, Van'tVeer LJ and Bernards R. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 8489–8493.
Henriksson M and Lüscher B. (1996). *Adv. Canc. Res.*, **68,** 109–182.
Hulboy DL and Lozano G. (1994). *Cell Growth Diff.*, **5,** 1023–1031.
Kadlec L and Pendergast A-M. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 12390–12395.
Kato GJ, Barrett J, Villa-Garcia M and Dang CV. (1990). *Mol. Cell. Biol.*, **10,** 5914–5920.
Kelekar A and Cole M. (1986). *Mol. Cell. Biol.*, **6,** 7–14.
Kretzner L, Blackwood EM and Eisenman RN. (1992). *Curr Top. Microbiol. Immunol.*, **182,** 435–443.
Land H, Parada LF and Weinberg RA. (1983). *Nature*, **304,** 596–602.
Lupas A. (1996). *Meth. Enz.*, **266,** 513–525.
Maruyama K, Schiavi SC, Huse W, Johnson GL and Ruley HE. (1987). *Oncogene*, **1,** 361–367.
Michalovitz D, Halevy O and Oren M. (1990). *Cell*, **62,** 671–681.
Negorev D, Reithman H, Wechsler-Reya R, Sakamuro D, Prendergast GC and Simon D. (1996). *Genomics*, **33,** 329–331.
O'Reilly DR, Miller LK and Luckow VA. (1992). In: *Baculovirus expression vectors: a laboratory manual*. WH Freeman and Co., Inc., New York.
Packham G and Cleveland JL. (1997). *Oncogene*, **15,** 1219–1232.
Phelps WC, Yee CL, Munger K and Howley PM. (1988). *Cell*, **53,** 539–547.
Prendergast GC. (1997). In: *Oncogenes as Transcriptional Regulators*. Yaniv M and Ghysdael J. (eds). Birkhauser Verlag: Boston, pp. 1–28.
Prendergast GC, Hopewell R, Gorham B and Ziff EB. (1992). *Genes Dev.*, **6,** 2429–2439.
Prendergast GC, Lawe D and Ziff EB. (1991). *Cell*, **65,** 395–407.
Prendergast GC and Ziff EB. (1991). *EMBO J.*, **10,** 757–766.
Ramjaun AR and McPherson PS. (1998). *J. Neurochem.*, **70,** 2369–2376.
Ruley HE. (1983). *Nature*, **304,** 602–606.
Sakamuro D, Elliott K, Wechsler-Reya R and Prendergast GC. (1996). *Nature Genet.*, **14,** 69–77.
Sakamuro D, Sabbatini P, White E and Prendergast GC. (1997). *Oncogene*, **15,** 887–898.
Schreiber-Agus N, Chin L, Chen K, Torres R, Rao G, Guida P, Skoultchi AI and De Pinho RA. (1995). *Cell*, **80,** 777–786.
Walker KK and Levine AJ. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 15335–15340.
Wechsler-Reya R, Elliott K, Herlyn M and Prendergast GC. (1997a). *Cancer Res.*, **57,** 3258–3263.
Wechsler-Reya R, Elliott K and Prendergast GC. (1998). *Mol. Cell. Biol.*, **18,** 566–575.
Wechsler-Reya R, Sakamuro D, Zhang J, Duhadaway J and Prendergast GC. (1997b). *J. Biol. Chem.*, **272,** 31453–31458.
Wigge P and McMahon HT. (1998). *Trends Neurosci.*, **21,** 339–344.
Yuan ZM, Huang Y, Fan MM, Sawyers C, Kharbanda S and Kufe D. (1996). *J. Biol. Chem.*, **271,** 26257–26460.
Zhang H and Prochownik E. (1997). *J. Biol. Chem.*, **272,** 17416–17424.
Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N and Harlow E. (1993). *Genes Dev.*, **7,** 1111–1125.

Genomics **56,** 51–58 (1999)
Article ID geno.1998.5709, available online at http://www.idealibrary.com on IDEAL®

The Murine *Bin1* Gene Functions Early in Myogenesis and Defines a New Region of Synteny between Mouse Chromosome 18 and Human Chromosome 2

Nien-Chen Mao,* Eirikur Steingrimsson,†,[1] James DuHadaway,* Wyeth Wasserman,‡ Joseph C. Ruiz,* Neal G. Copeland,† Nancy A. Jenkins,† and George C. Prendergast*,[2]

*The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104; †Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI–Frederick Cancer Research and Development Center, Frederick, Maryland 21702; and ‡Smith Kline Beecham Research Laboratories, King of Prussia, Pennsylvania 19190

Received September 2, 1998; accepted December 4, 1998

We cloned and functionally characterized the murine *Bin1* gene as a first step to investigate its physiological roles in differentiation, apoptosis, and tumorigenesis. The exon–intron organization of the ≥55-kb gene is similar to that of the human gene. Consistent with a role for *Bin1* in apoptosis, the promoter included a functional consensus motif for activation by NF-κB, an important regulator of cell death. A muscle regulatory module defined in the human promoter that includes a consensus recognition site for myoD family proteins was not conserved in the mouse promoter. However, *Bin1* is upregulated in embryonic development by E10.5 in myotomes, the progenitors of skeletal muscle, supporting a role in myogenesis and suggesting that the mouse and human genes may be controlled somewhat differently during development. In C2C12 myoblasts antisense *Bin1* prevents induction of the cell cycle kinase inhibitor p21WAF1, suggesting that it acts at an early time during the muscle differentiation program. Interspecific mouse backcross mapping located the *Bin1* locus between *Mep1b* and *Apc* on chromosome 18. Since the human gene was mapped previously to chromosome 2q14, the location of *Bin1* defines a previously unrecognized region of synteny between human chromosome 2 and mouse chromosome 18.

INTRODUCTION

The identification and functional analysis of tumor suppressor genes are major goals of cancer research. Bin1 is a tumor suppressor in breast and prostate carcinoma that was identified through its ability to interact with the transcriptional regulatory domain of the Myc oncoprotein (Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1997a). Bin1 inhibits the oncogenic and transcriptional properties of Myc (Sakamuro *et al.,* 1996; Elliott *et al.,* in press) but it can also inhibit cell growth by Myc-independent mechanisms (Elliott *et al.,* in press). A necessary role has been defined for Bin1 in differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998). Recent work has identified Abl as a second oncoprotein that interacts with Bin1, through its SH3 domain (Kadlec and Pendergast, 1997) that is dispensible for Myc interaction (Sakamuro *et al.,* 1996). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein that is a paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.,* 1994; Dropcho, 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.,* 1993; Crouzet *et al.,* 1991). Amphiphysin has been implicated in receptor-mediated endocytosis (David *et al.,* 1996; Wigge *et al.,* 1997b), and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin-related protein (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997), have been reported to interact with amphiphysin and to influence endocytosis (Wigge *et al.,* 1997a; Owen *et al.,* 1998). Taken together, the results suggest that Bin1 is a nucleocytoplasmic adaptor that participates in a signaling pathway(s) linking membrane trafficking with gene and cell cycle regulatory events.

The human *BIN1* gene has been cloned and characterized (Wechsler-Reya *et al.,* 1997b). It is ubiquitously expressed and extensively alternately spliced with highest expression in skeletal muscle. *BIN1* is located at chromosome 2q14 (Negorev *et al.,* 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.,* 1996). We cloned the mouse *Bin1* gene (alternate symbol *Amphl*) as a prerequisite to generating homozygous null animals that

[1] Present address: Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Iceland, Vatnsmyrarvegi 16, 101 Reykjavik, Iceland.

[2] To whom correspondence should be addressed. Telephone: (215) 898-3792. Fax: (215) 898-2205. Email: prendergast@wista.wistar.upenn.edu.

0888-7543/99 $30.00

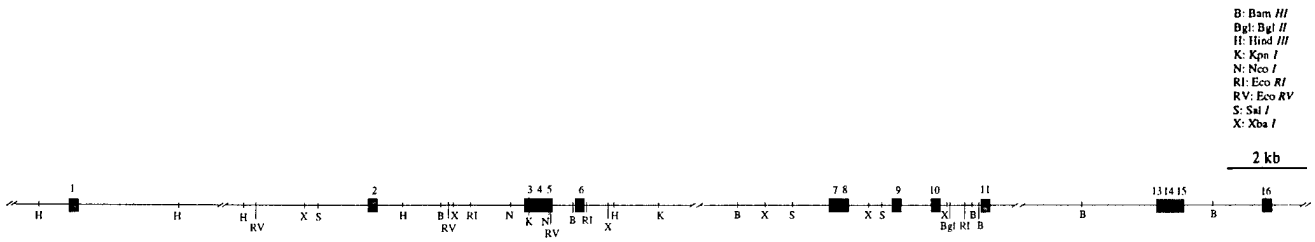


FIG. 1. Physical map of the mouse *Bin1* gene. A restriction map of four contiguous regions of the gene derived from subclones from a single BAC clone is shown. Exons located by Southern analysis with a murine cDNA and limited DNA sequencing were numbered by comparison to the human gene (Wechsler-Reya *et al.,* 1997b).

will allow investigations of its physiological functions. In this study, we defined the exon–intron organization, promoter, and muscle-specific expression of mouse *Bin1.* We also obtained evidence supporting a function for *Bin1* in regulating apoptosis and myogenesis. Finally, we mapped the chromosomal location of the gene to mouse chromosome 18, a locus that defines a new region of synteny with human chromosome 2.

MATERIALS AND METHODS

Gene cloning and characterization. Three clones were isolated from a murine 129/Sv BAC library (Genome Systems) by hybridization with a full-length human cDNA (Sakamuro *et al.,* 1996). Restriction fragments were subcloned into pKS II (−) (Stratagene) and analyzed by extensive restriction mapping and Southern analysis with human Bin1 cDNA probes. The DNA sequences of exon-containing subclones were determined using an automated DNA sequencer. Primer sequences were derived from a full-length murine cDNA, SH3P9 (Sparks *et al.,* 1996), which encodes the ubiquitously expressed splice form of Bin1 lacking exon 10 sequences (Bin1-10) (Wechsler-Reya *et al.,* 1997b,1998). Exon 16 was derived from a mouse ES cell genomic fragment generated by PCR, using primers in exon 15 and 16, because none of BAC clones included this exon. Exon 16 sequences shown in Fig. 2 include sequences from the ES subclone as well as from SH3P9 (Sparks *et al.,* 1996).

DNA sequence analysis. The sequence data were assembled manually with assistance from MacVector and AssembLIGN software. Exons were defined by alignment and comparison to the human *BIN1* gene (Wechsler-Reya *et al.,* 1997b) with additional alignments to Bin1 expressed sequence tags in GenBank. Similarity between the mouse and the human coding regions was computed using the BLASTN and TBLASTN algorithms. Promoter sequences were aligned using CLUSTAL W (1.7) and analyzed with a muscle module detection algorithm (Wasserman and Fickett, 1998) that identifies clustered transcription factor binding sites characteristic of muscle-specific promoters.

Immunohistochemistry. Mouse embryos (10.5 days) were fixed for 24 h in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Five micrometer-thick sections were cut and mounted on Snowcoat X-tra Micro slides (Surgipath), air-dried, and heat-fixed for 30 min at 56°C. Slides were deparaffinized in xylene twice for 10 min each and then rehydrated in decreasing percentages of ethanol, starting at 100% and ending in PBS. Endogenous peroxidase was quenched by incubating for 15 min in 1% H_2O_2 in methanol followed by PBS washing. Slides were then placed in a 600-ml beaker in a slide rack containing 500 ml of 10 mM citrate buffer (pH 8.5), covered with plastic wrap, and microwaved for 5-min intervals for a total of 10 min at the highest power setting (Catoretti *et al.,* 1992). After slides were cooled in the citrate buffer for 20 min, antibody staining was performed essentially as described (Sakamuro *et al.,* 1996). Briefly, tissue was blocked with 10% goat serum and incubated for 30 min with a 1:1500 dilution of Bin1 99I monoclonal antibody from ascites (Wechsler-Reya *et al.,* 1997a). The primary antibody was visualized by a 30-min incubation with biotin-conjugated goat anti-mouse antibody, a 30-min incubation with peroxidase-conjugated streptavidin, and a brief treatment with diaminobenzidine. Before mounting, slides were counterstained with an acidified solution of the cytoplasmic dye light green. Stained embryos were photographed using Kodak T64 film on a Leitz microscope at 80× or 400× magnification.

Western analysis. C2C12 cells expressing antisense Bin1 or containing vector only were cultured in growth or differentiation medium and cell extracts were prepared and processed for Western analysis as described (Wechsler-Reya *et al.,* 1998). Blots were probed with rabbit anti-p21 polyclonal antibody C21 (Santa Cruz Biotechnology) or with murine anti-myosin monoclonal antibody MF20 (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA). HRP-coupled goat anti-mouse or anti-rabbit IgG (BMB) was used with a commercial chemoluminscence kit (Pierce) to develop the blots. Equal protein loading per lane was subsequently confirmed on blots by staining with Ponceau S.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). Haplotype analysis of 164 N_2 mice was performed to map the *Bin1* locus; for gene order determination up to 193 mice were typed for some pairs of markers. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, a 1.4-kb *Eco*RI/*Hin*dIII fragment of mouse *Bin1* cDNA, was labeled with [α-^{32}P]dCTP using a random priming kit (Amersham); washing was performed to a final stringency of 1.0 × SSCP, 0.1% SDS at 65°C. The probe detected 13.0-, 10.5-, 8.0-, 5.0-, 4.6-, and 0.5-kb *Bam*HI fragments in C57BL/6J DNA and 10.0-, 8.0-, 6.6-, 5.4-, 4.7-, 2.7-, and 0.9-kb fragments in *Bam*HI-digested *M. spretus* DNA. The presence or absence of the 6.6-, 5.4-, 2.7-, and 0.9-kb *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. A description of probes and restriction fragment length polymorphisms (RFLPs) for loci linked to *Bin1,* including desmoglein-3 (*Dsg3*), meprin1, β subunit (*Mep1b,*) and the adenomatosis polyposis coli gene (*Apc*) has been reported previously (Gorbea *et al.,* 1993; Ishikawa *et al.,* 1994). Recombination distances and gene orders were determined using Map Manager, version 2.6.5 (Manly, 1993); gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS AND DISCUSSION

Structure of the Murine Bin1 Gene

A physical map of *Bin1* was determined from a single BAC genomic clone that included the entire locus within an ~100-kb insert (see Fig. 1). The relative location of each exon was determined by Southern analysis, and exon and proximal intron sequences were determined by DNA sequencing (see Fig. 2). The mouse

No.	EXON	INTRON
1	CTCACTCGCTCTCCCCGCGCACGCTCCGTCTCCGTCAGTCCCCTGAGCTG TTCTAGTGCGCGGCGTGGAGCCAGGGCTCAGGCTGGTGGAGCGGCCGGGG CTGGAGGCTGGGAGTGCGGCGCGCACGGCCTCCCCGCGCCATTATCCGCG CTCGCTTCGGGCGAGGCCGGCGCCAGGATGGCAGAGATGGGGAGCAAGGG GGTGACGGCGGGGAAGATCGCCAGCAACGTACAGAAGAAGCTGACCCGAG CGCAGGAGAAG	gtgagtgaacggtaaacctgccacacctctcgcccctacccccgggatct ---- > 10 kb ---- ccggcccctggcttctcaaggtgatgttgcccnttntctctgtgcggcag
2	GTCCTGCAGAAACTGGGGAAGGCGGACGAGACGAAGGACGAGCAGTTTGA GCAGTGTGTCCAGAACTTCAATAAGCAGCTG	gtgagtgtttatggaggtgggacagcgtttgctaggtaggagatggtgag ---- ~ 6 kb ---- ttggtgacagggtccccaggacctgaccttgttcttggctttttctggcag
3	ACAGAGGGTACCCGGCTGCAGAAGGATCTTCGGACCTATCTGGCTTCTGT TAAAG	gtagggtacgtctctctgtaaggatttggggctgtcaagctgaggtgggc ---- ~ 150 bp ---- tgtatgagcccgagtggcctggcagggatgaagcaaacaagattgcagag
4	CGATGCACGAAGCCTCCAAGAAGCTGAGTGAGTGTCTTCAGGAGGTGTAT GAGCCCGAGTGGCCTGGCAGGGATGAAGCAAACAAGATTGCAGAG	gtaagcatgggtgggtgcccttggttcttcccccaaggccctttttggctt ---- ~ 300 bp ---- tgcagacagtgtggcttagctctacaaacgctcctgtttctatgtttcag
5	AACAATGACCTACTCTGGATGGACTACCACCAGAAGCTGGTGGACCAGGC TCTGCTGACCATGGACACCTACCTAGGCCAGTTCCCTGATATCAAG	gtaagaaacctctgggcccattgtcttgcttgggttggagcttgtggaag ---- ~ 1.3 kb ---- gggatcttggcaccaggccccanngatctctcctcctctctgctccctag
6	TCGCGCATTGCCAAGCGGGGGCGGAAGCTGGTGGACTATGACAGTGCCCG GCACCACTATGAGTCTCTTCAAACCGCCAAAAAGAAGGATGAAGCCAAAA TTGCCAAG	gttcccanctgtgggtggggatggtgctgantccagngccacatanaaca ---- > 10 kb ---- ggtagagttccacacagacgctgacgtaccccactgcctctccatcccag
7	GCAGAAGAGGAGCTCATCAAAGCCCAGAAGGTGTTCGAGGAGATGAACGT GGATCTGCAGGAGGAGCTGCCATCCCTGTGGAACAG	gtaaagtcaggaggggcccaggaacctggcgttcagcctggccctgtgtc ---- ~ 200 bp ---- catcccgtcgcatatggttctcaccatgtcacctcctatctctctggcag
8	CCGTGTAGGTTTCTATGTCAACACGTTCCAGAGCATCGCGGGTCTGGAGG AAAACTTCCATAAAGAGATGAGTAAG	gtaggccaggggactgggctgtgcaaaggatcagtcagaggcagggatg ---- ~ 2 kb ---- tgacgaagatgctcgtccaagcctgcttctcttcactcttcctcctgcag
9	CTCAATCAGAACCTCAATGATGTCCTGGTCAGCCTAGAGAAGCAGCACGG GAGCAACACCTTCACAGTCAAGGCCCAACCCAG	gtaggttagggcagggagggtgaggtcagtggggccctgtggcatgatgg ---- ~ 1.5 kb ---- ttcctagctttcttccaaatgaagcatccacactccaaccatccccacag
10	AAAGAAAAGTAAACTGTTTTCGCGGCTGCGCAGAAAGAAGAACAG	gtaccgccttgagtgcagtgccacgggcctctgggccccgccctgactgc ---- ~ 1.9 kb ---- ctggctctttgttctgttgataccactctcggtctgtccttcttttacag
11	TGACAATGCCCCTGAGAAAGGGAACAAGAGCCCGTCACCTCCTCCAGATG GCTCCCCTGCTGCTACCCCTGAGATCAGAGTGAACCATGAGCCAGAGCCG GCCAGTGGGGCCTCACCCGGGGCTACCATCCCCAAGTCCCCATCTCAG	gtaggcacgactgttatctctatgtcctggttttcttctctttctctttc ---- > 6 kb ---- ggatgcatcctgctctgtatctgatcccttgctggcattttatgttgcag
13	CCAGCAGAGGCCTCCGAGGTGGTGGGTGGAGCCCAGGAGCCAGGGGAGAC AGCAGCCAGTGAAGCAACCTCC	gtaagacggcaggggccgggccctgtttttttcttccctgttgtctgtctg ---- ~ 300 bp gcttttcctacatggccattggtccagctgactcatcccctatccctcag
14	AGCTCTCTTCCGGCTGTGGTGGTGGAGACCTTCTCCGCAACTGTGAATGG GGCGGTGGAGGGCAGCGCTGGGACTGGACGCTTGGACCTGCCCCCGGGAT TCATGTTCAAG	gtgagcgtaggctagccaactctgtagcctttgtctccggtgccttgggg ---- ~ 200 bp tggttaagatgggggaatagcccccctganatgccttcttaatttntacag
15	GTTCAAGCCCAGCATGATTACACGGCCACTGACACTGATGAGCTGCAACT CAAAGCTGGCGATGTGGTGTTGGTGATTCCTTTCCAGAACCCAGAGGAGC AG	gtgaacaagggtgtggggaatcccctggctgctgatgcaatggtgggcat ---- ~ 3 kb---- tggtgagtgctgtgtggtgctcctgtgttagccatgctctgttggccccag
16	GATGAAGGCTGGCTCATGGGTGTGAAGGAGAGCGACTGGAATCAGCACAA GGAACTGGAGAAATGCCGCGGCGTCTTCCCGGAGAATTTTACAGAGCGGG TACAGTGACGGAGGAGCCTTCCGGAGTGTGAAGAACCTTTCCCCCAAAGA TGTGTG	

FIG. 2. Exon–intron structure. Exon and proximal intron sequences for the ubiquitously expressed exons within the Bin1 gene are shown. The figure is read from left to right with complete exon sequences shown on the *left* and introns following on the *right*. Register is 50 nt per line. Sizes of intron gaps are approximate based on estimations from restriction mapping.

gene is similar in both structure and organization to the human gene (Wechsler-Reya *et al.,* 1997b), including conservation of a large intron 1 (>20 kb) and a region of ~35 kb that includes the remaining exons. Exons were numbered by reference to the human gene. The mouse *Bin1* gene is ≥55 kb in length, which is similar to the ≥54-kb size of the human gene (Wechsler-Reya *et al.,* 1997b). A full-length mouse Bin1 message, which has been described [SH3P9; (Sparks *et al.,* 1996)], encodes the ubiquitously expressed Bin1 splice form Bin1-10 that includes exons 1–9, 11, and 13–16 (Wechsler-Reya *et al.,* 1997b,1998). The mouse and human coding sequences are ~89% identical at the nucleotide level (mouse nt 41–1461) and ~95% identical at the amino acid level (comparing the ubiquitously expressed Bin1-10 splice isoforms). Proximal intron sequences for each exon were generally highly conserved as well. The BAR (Bin1/Amphiphysin/Rvs167-related) region (Sakamuro *et al.,* 1996) encoded by exons 1–8 includes sequences that are conserved in amphiphysin and conserved in organization to the human Bin1 gene. Exons 9–11 encode the unique region of Bin1 that is not conserved in amphiphysin or RVS167. Exon 9 encodes the unique-1 (U1) region that includes sequences crucial for Bin1 to suppress malignant cell transformation by adenovirus E1A or mutant p53 (Elliott *et al.,* submitted for publication). Exon 10 encodes the unique-3 region (U3) of Bin1 which is alternately spliced following differentiation of C2C12 mouse myoblasts *in vitro* (Wechsler-Reya *et al.,* 1998). U3 was initially thought to encode a nuclear localization signal (Sakamuro *et al.,* 1996) but later investigations argued against this likelihood (Wechsler-Reya *et al.,* 1998). Exon 11 encodes the proline-rich unique-2 (U2) region, which can serve as a pseudosubstrate for the Bin1 SH3 domain (D. Sakamuro, unpublished observations). Murine exons corresponding to brain-specific exons 12A–12D in the human gene (Wechsler-Reya *et al.,* 1997b) were not analyzed in this study. Exons 13 and 14 encode sequences homologous to the Myc-binding domain in human Bin1 (Sakamuro *et al.,* 1996). Exon 13 is alternately spliced in an unregulated fashion in adult and embryonic mouse tissues (Wechsler-Reya *et al.,* 1998; Wechsler-Reya *et al.,* 1997b,).

```
mouse   ---AATGGAAAAACGGAGTGGTTAGTNAGCATGGGNTAGGCA----AAGAGAAAGGGACA  -484
human   CGAACGGGGAAGACCAAGCACCGGTTGGTACTGGGTTAGGCGCCGTAGGGCAAAGATGTG  -534

mouse   GAGA--------------------------------AAAAGCCATAGGCCACAGGGTGCAGC  -455
human   GAGATGTCCCGGAGGCGCCTAGGGTATCCGGGCGAAAACCCGAGGGCCGAAGGCTG--GG  -477

mouse   AGGAGGCGGA----------CGTGGATGNTAGCAGGAGGGAAATCCTTGGTA------GG  -412
human   AGGAGGCGGAGCGTCGGGCACCGGGCACCGGGCGGGAGGTGAGCCCCTGGAAAAGGAGGG  -418

mouse   GACTT-----------TCCCAGCCCGCGGGGGANTTTGGGAGTCCAGGGCCACGCANGCG  -364
human   GACTCCGGGCGCGTTCTCCCAGCAGCCGCGGCTCCTCTG---TTCAGGGCCGCGCCCCC-  -363

                              Mef2
mouse   TNTATCCCTGCACATTGTCTTTGATTTTTAGAAAGCACTGGACTCCTTCACCTGGT-TAC  -306
human   ---TTCGCCGCACTTTTTCTTTGATTTC--GAAAGCACTCCTCTCCTCCACCTAGTCTCC  -309

           Tef
mouse   ATTCTAGAGTTGCAGAGGTAT--CTGTTTGGAAGGAGAACTTACGCGGTGACACTGAATT  -249
human   TTTCCTGGGTTGCAGGAGAGTTACTGCTTTGC-GGGGAAAGAACAAGACGCCA-------  -268

                                          Myf/MyoD          NF-KB
mouse   GGGGACAGCATAGGTAGTTCCCATTCCCAGGCGAAGTTGTAAGCGCATTTGGGGAGTCCC  -190
human   GG--CCGGCGGAT-TAGTCCCCGCCCCGGGGCGGTGCAGCTGGAGCGTCAGGGGAGTCCC  -212

mouse   TGAC-CTGCAGCCCCCAGTGCCCGCCCTCCAGGATCCCTCCTC------CTGGGCGGTGA  -138
human   GCTCGCCGCAGCCCCAGCGCCGCGCGCGCC--CATCCATCCTAGAAGGACCTGGCGGTG-  -156

                                  SRF                    Sp1
mouse   GATCCAGATCCCAGAATGGCCCTTTAAAAGGCAGTGTCGTGTCCGGAGAGGGCGGGCTGG  - 79
human   ---CCGGCGCCCGGAGTGGCCCTTTAAAAGGCAGCTTATTGTCCGGAGGGGGCGGGCGGG  -100

                              TBP
mouse   GGGCCACTGACCCGCCC-GCGGCTGGTCCTTTTTCCCCGCCCT-------TCCCTCCTCC  - 28
human   GGGCGCCGACCGCGGCCTGAGGCCCGGCCCCTCCCCTCTCCCTCCCTCTGTCCCCGCGTC  - 40

mouse   TTTGGCTCCCTCCCTCCCTGGAT------------CCCCGCGTTG                 +  7
human   GCTCGCTGGCTAGCTCGCTGGCTCGCTCGCCCGTCCGGCGCAC                  +  4
                                           ••+1
```

FIG. 3. Structure and conserved regulatory elements of the mouse Bin1 promoter. The DNA sequence of ~0.5 kb of the 5′ flanking region of the mouse gene is shown and aligned with the human promoter. Sequence alignment was performed using CLUSTAL W (1.7) except for the sequences immediately surrounding the human gene cap site (dot) and 5′ end of the mRNA (double underlining), which was aligned by visual inspection. Mouse sequences were numbered by comparison to the human promoter (Wechsler-Reya *et al.,* 1997b). A muscle regulatory module identified by a sequence analysis algorithm (Wasserman and Fickett, 1998) in the human promoter between −330 and −125 is indicated by dotted underlining. Consensus DNA binding sites for various transcription factors identified through this analysis or through visual inspection are noted by solid underlining. Mef2 and Tef consensus sites in the mouse were not conserved in the human promoter, whereas a strong Myf/MyoD consensus site in the human was not conserved in the mouse. A site analogous to the TBP binding site noted in the human promoter (Wechsler-Reya *et al.,* 1997b) was also not detected in the mouse sequence.

Exons 15 and 16 encode the Src homology 3 domain of Bin1, a feature that is shared with amphiphysin and RVS167 (Sakamuro *et al.,* 1996) and that is necessary for interaction with c-Abl and dynamin (Kadlec and Pendergast, 1997; Owen *et al.,* 1998).

Conserved Features of the Bin1 Promoter Suggest Roles in Myogenesis and Apoptosis

The DNA sequence of the 5′ flanking region upstream of exon 1 was determined, and this region was analyzed and compared with the human promoter (see Fig. 3). There was significant conservation between the mouse and the human 5′ flanking regions within 400 bp of exon 1, consistent with the identification of the mouse *Bin1* promoter. Bin1 is expressed robustly in skeletal muscle from adult mouse and human (Sakamuro *et al.,* 1996), so the 5′ flanking regions of each gene were analyzed using algorithms that identify transcription factor consensus binding sites and muscle regulatory modules (Wasserman and Fickett, 1998). A muscle regulatory module was identified in the human promoter between −330 and −125 (Fig. 3, dotted underline) but an analogous module was not detected in the mouse promoter. This absence suggested that there may be some difference in how the human and mouse genes are regulated during differentiation. Consistent with the latter possibility, the mouse region also lacked a CpG island that is present in the human promoter (Wechsler-Reya *et al.,* 1997b). The muscle regulatory sites identified in the human module included myogenin/myoD, Sp1, and serum response factor (SRF). The role of the myoD family b/HLH transcription factors in directing muscle differentiation is well known. SRF was initially identified as

an activator of the c-*fos* promoter but was later found to be crucial for regulating skeletal muscle-specific and smooth muscle-specific gene expression (Duprey and Lesens, 1994). In the 5′ flanking region of the mouse gene, the Sp1 and SRF sites were conserved but not the myogenin/myoD site, pointing to another difference with the human promoter. In place of the myogenin/myoD site were weak sites for Mef2 and Tef. Mef2 is a MADS-box transcription factor that is required for muscle development (Olson *et al.,* 1995). Since *Bin1* must be upregulated for differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), the Mef2 site may be relevant to this event because it has also been shown to be required for C2C12 differentiation (Ornatsky *et al.,* 1997). The Mef site may also be relevant to the high-level expression of *Bin1* in adult brain (Butler *et al.,* 1997; Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1997b) and in PC12 cells (R. Wechsler-Reya and G.C.P., unpublished results) since Mef2 isoforms are strongly expressed in neuronal cells (Lyons *et al.,* 1995). The Tef site may be relevant to housekeeping as well as muscle-specific regulation of Bin1. Tef was originally identified as an SV40 enhancer-binding factor but was subsequently discovered to be an important factor in cardiac muscle-specific gene regulation (Farrance *et al.,* 1992). Sp1 and Tef sites are proximal in the SV40 promoter so interactions between these factors may be involved in the basal but ubiquitous *Bin1* expression seen in tissues outside of muscle and brain (Wechsler-Reya *et al.,* 1997b). Taken together, the data suggested that SRF and Sp1 directed muscle-specific expression of *Bin1* with additional contributions from myogenin/myoD in human and from Tef and Mef2 in mouse.

One notable feature of the *Bin1* promoter identified in this study was the presence of an evolutionarily conserved strong consensus binding site for NF-κB. This finding was interesting because of evidence that Bin1 has a positive role in c-Myc-mediated apoptosis and that Bin1 can drive apoptosis of tumor cells that contain deregulated c-Myc (D. Sakamuro, K. Elliott, K. Ge, J. Duhadaway, D. Ewert, and G.C.P., manuscripts in preparation). NF-κB has important roles in oncogene-mediated cell transformation (Mayo *et al.,* 1997; Reuther *et al.,* 1998) and apoptosis (Baichwal and Baeuerle 1997). The likelihood that the NF-κB site in the Bin1 promoter is functional was supported by the observations that (1) Bin1 message levels were increased by TNF-α, which stimulates NF-κB activity, and that (2) a Bin1 promoter-reporter gene could be activated several-fold by TNF-α or RelA/p50 and c-Rel/p50 in transient cotransfection assays (data not shown). In future work, it will be important to determine whether Bin1 mediates certain NF-κB responses in apoptosis, for example, those activated by tumor necrosis factor, and whether the Myc-Bin1 system may modulate the ability of NF-κB to control apoptosis.

Bin1 Is Upregulated during Development by E10.5 in Myotomes

The inability to identify a muscle regulatory module in the mouse *Bin1* promoter prompted us to investigate the expression of *Bin1* during muscle development. Myogenesis initiates during development in somites, segmented paraxial mesoderm that is arrayed along the dorsal axis alongside the developing central nervous system. The dorsal part of the somite includes the myotome, which is the progenitor of skeletal muscle. Early stages of myogenesis are apparent in E10.5 myotomes because myf5 and myoD have been switched on and elongation of cells destined to become muscle can be seen. We performed an immunohistochemical analysis of E10.5 embryos with a Bin1 monoclonal antibody (Wechsler-Reya *et al.,* 1997a) to determine whether *Bin1* was switched on at this stage. In sagittal sections, strong staining was detected in elongated cells present in a dorsally located segmented pattern consistent with somites (see Figs. 4A and B). The cytoplasmic staining pattern was consistent with that seen following *in vitro* differentiation of C2C12 myoblasts, when Bin1 is exported from the nucleus to the cytosol (Wechsler-Reya *et al.,* 1998). In transverse sections, staining was confined to a medial part of a dorsolateral segment of the myotome (see Fig. 4C, arrowhead, and Fig. 4D). Staining was specific in the somite region inosfar as there was no significant staining of the adjacent sclerotomes, which are the progenitors of skeletal bone. The data indicated that *Bin1* was activated during myogenesis even though its promoter lacked a consensus DNA binding site for the important myoD family of muscle determination factors (Molkentin and Olson, 1996). Mef2 is expressed before E10.5 in the myotome (Edmondson *et al.,* 1994) so this factor may be responsible for activating the *Bin1* gene at this time. We concluded that despite the absence of a myoD family consensus binding site in its promoter, *Bin1* was activated at an early stage of myogenesis.

Bin1 Functions at an Early Time during Myoblast Differentation

In previous work, we showed that Bin1 is induced and has a necessary role during differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), a model for muscle differentiation *in vitro.* The immunochemical results above validated the induction of Bin1 seen in C2C12 and raised the question of when Bin1 acts during myoblast differentation. We have shown that C2C12 cells expressing antisense Bin1 do not exit the cell cycle and differentiate following serum deprival (Wechsler-Reya *et al.,* 1998). An important early event in C2C12 differentiation that leads to cell cycle inhibition is induction of the cell cycle kinase inhibitor $p21^{WAF1}$ (Walsh and Perlman, 1997). Therefore, we examined the regulation of $p21^{WAF1}$ or myosin, a marker for biochemical differentiation, in antisense or control C2C12 cell lines generated previously.

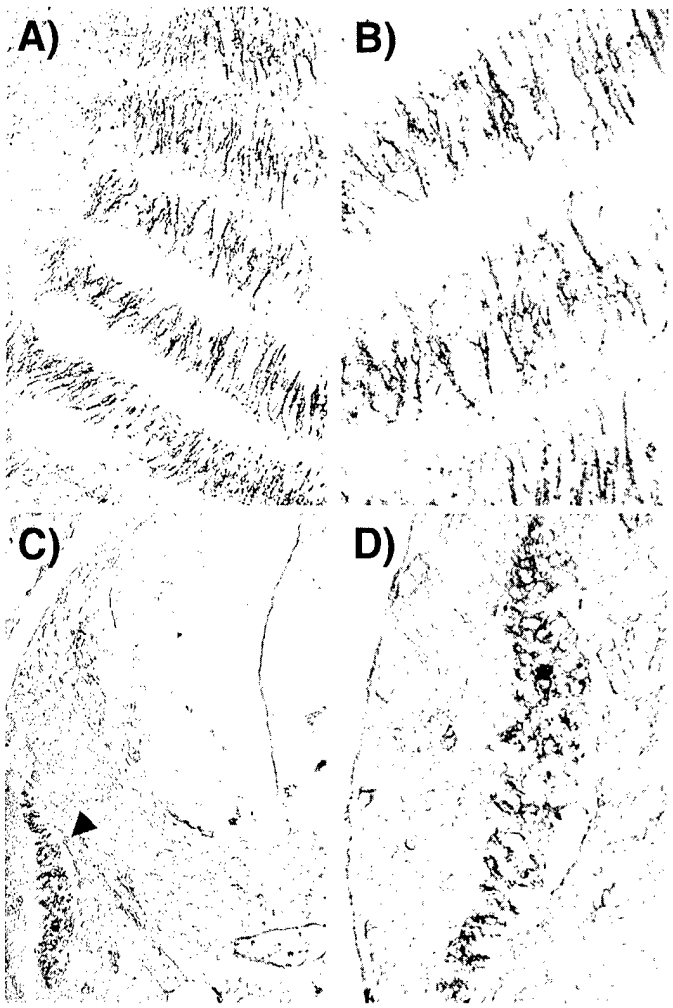


FIG. 4. *Bin1* is specifically upregulated in myotomes by E10.5 during murine development. E10.5 embryos were processed for Bin1 immunohistochemistry as described under Materials and Methods. (**A**) Sagittal section illustrating expression of *Bin1* in elongating myoblasts in somites, 80×. (**B**) Same section as above, 400×. (**C**) Transverse section illustrating expression of *Bin1* in the myotome but not the sclerotome of a somite (arrowhead), 80×. Orientation is provided by the neural tube seen in the upper right corner of the figure. Expression of *Bin1* in endothelial cells (bottom right side of the figure) and in certain neurons in the neural tube, a phenomenon also noted in the brain (data not shown), is also illustrated in this figure. (**D**) Same section as above, 400×.

Western analysis of extracts isolated at various times after induction of differentiation by serum deprival showed that $p21^{WAF1}$ was not appropriately upregulated in antisense cells (see Fig. 5). In control cells, $p21^{WAF1}$ levels steadily increased from a basal level of expression starting at day 1 after serum deprival. Myosin expression indicative of complete biochemical differentiation was first detected at day 3 in these cells. In contrast, in cells expressing antisense Bin1, $p21^{WAF1}$ was undetectable in undifferentated cells (day 0) and was only transiently induced on day 1 after differentiation was induced. As seen before, these cells did not proceed to express myosin and biochemically differentiate (Wechsler-Reya *et al.,* 1998), presumably because $p21^{WAF1}$ was not appropriately upregulated such that cells could exit the cell cycle. We concluded that Bin1 functioned at an early stage of myoblast differentiation, at a point required to sustain activation of $p21^{WAF1}$ and subsequent cell cycle exit.

Bin1 Is Located within the Proximal Region of Mouse Chromosome 18

The chromosomal location of *Bin1* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). To identify informative RFLPs for gene mapping, the mouse *Bin1* cDNA was used as a probe in Southern blot analysis of C57BL/6J and *M. spretus* genomic DNAs digested with several restriction enzymes (see Materials and Methods). The inheritance of the *M. spretus*-specific alleles was followed in backcross mice, and the strain distribution pattern of the RFLP was determined to position the locus on the interspecific backcross map. The mapping results indicated that the *Bin1* locus is located in the proximal region of chromosome 18 (see Fig. 6), 0.5 cM proximal to *Apc.* The same results were obtained by using *Hin*cII polymorphisms and by using the human *BIN1* cDNA as a probe to follow *Taq* I polymorphisms (data not shown). We compared our interspecific map of chromosome 18 with composite mouse linkage maps that report the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME).

In humans, *BIN1* has been mapped to chromosome 2q14 by fluorescence *in situ* hybridization and by PCR analysis of somatic cell hybrids (Negorev *et al.,* 1996), but synteny has not been reported previously between human chromosome 2q14 and mouse chromosome 18. However, in the mouse, synteny has not been deter-

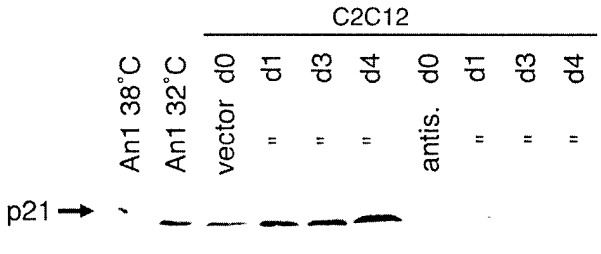


FIG. 5. Impaired activation of $p21^{WAF1}$ in myoblasts whose differentiation is blocked by antisense Bin1. Cell extracts were prepared from control or antisense Bin1-expressing C2C12 murine myoblasts (Wechsler-Reya *et al.,* 1998). Cells were cultured in growth medium (day 0, d0) or in differentiation medium for various times (d1,d3, and d4) before extract preparation. Western blotting was performed using anti-p21 and anti-myosin antibodies. A control for p21 induction was provided by the rat cell line BRK/An1, which harbors a temperature-sensitive p53 mutant; in this cell lines p21 is induced by activation of wildtype p53 at 32°C but not mutant at 38°C.

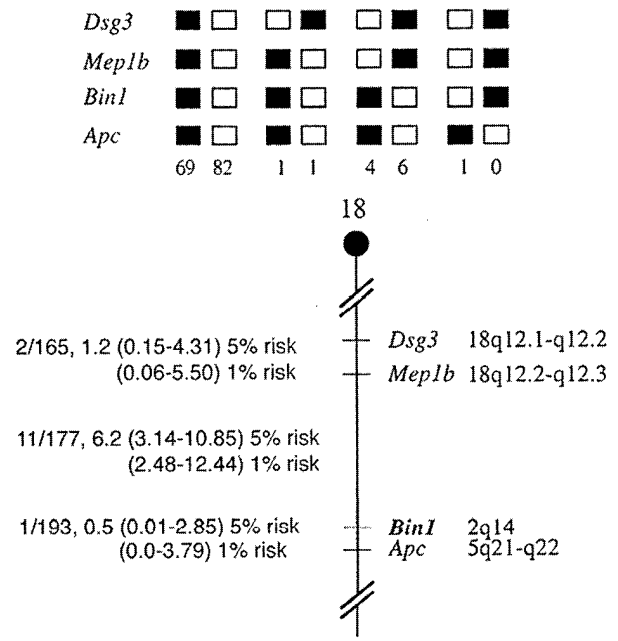


FIG. 6. Partial chromosome linkage map showing the mouse chromosomal location of *Bin1* as determined by interspecific backcross analysis. (**Top**) Segregation patterns of *Bin1* and flanking genes. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. Black boxes indicate the presence of a C57BL/6J allele, and white boxes indicate the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column, for a total of 164 mice analyzed for the segregation analysis of *Bin1*. (**Bottom**) Gene order analysis. Data from up to 193 mice were used to generate the partial chromosome linkage map of chromosome 18, which indicated the location of *Bin1* in relation to linked genes. To the left of the chromosome map is shown the number of recombinant N_2 animals over the total number of animals typed, with the recombination frequencies for each pair expressed as genetic distances in centimorgans (with confidence intervals at the 5 or 1% risk level). The positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

mined in the 6.7-cM region between loci that flank *Bin1* and have been mapped in both species (*Mep1b* and *Apc*). As discussed above, the human and mouse genes share significant similarity, and all the *Bin1* polymorphisms that we followed in backcross mice fell into the same region on mouse 18. Therefore, we conclude that the *Bin1* locus truly defines a new region of synteny.

ACKNOWLEDGMENTS

We are grateful to Brian Kay for providing the SH3P9 cDNA, which encodes a full-length murine Bin1 splice form lacking exon 10 sequences (Bin1-10). We thank Roberto Buccafusca, Linda S. Cleveland, and Debra J. Gilbert for excellent technical assistance. This research was supported by grants from the National Cancer Institute and the DHHS (N.C. and N.J.) and from the ACS and the U.S. Army Breast Cancer Research Program (G.C.P.). G.C.P. is a Pew Scholar in the Biomedical Sciences and the recipient of awards from the Association for the Cure of Cancer of the Prostate (CapCURE).

REFERENCES

Baichwal, V. R., and Baeuerle, P. A. (1997). Activate NF-kappa B or die? *Curr. Biol.* **7:** R94–R96.

Bauer, F., Urdaci, M., Aigle, M., and Crouzet, M. (1993). Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* **13:** 5070–5084.

Butler, M. H., David, C., Ochoa, G.-C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O., and De Camilli, P. (1997). Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/RVS family, is concentrated in the cortical cytomatrix of axon initial segments and nodes of Ravier in brain and around T tubules in skeletal muscle. *J. Cell Biol.* **137:** 1355–1367.

Catoretti, G., Dominoni, F., Fusilli, F., and Z. O. (1992). Microwave oven irradiation vs trypsin digestion for antigen unmasking in fixed, paraffin embedded material. *Histochem. J.* **24:** 594.

Cher, M. L., Bova, G. S., Moore, D. H., Small, E. J., Carroll, P. R., Pin, S. S., Epstein, J. I., Isaacs, W. B., and Jensen, R. H. (1996). Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res.* **56:** 3091–3102.

Copeland, N. G., and Jenkins, N. A. (1991). Development and application of a molecular genetic linkage map of the mouse genome. *Trends Genet.* **7:** 113–118.

Crouzet, M., Urdaci, M., Dulau, L., and Aigle, M. (1991). Yeast mutant affected for viability upon nutrient starvation: Characterization and cloning of the RVS161 gene. *Yeast* **7:** 727–743.

David, C., McPherson, P. S., Mundigl, O., and de Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA* **93:** 331–335.

David, C., Solimena, M., and De Camilli, P. (1994). Autoimmunity in stiff-man syndrome with breast cancer is targeted to the C-terminal regulation of human amphiphysin, a protein similar to the yeast proteins, Rvs161 and Rvs167. *FEBS Lett.* **351:** 73–79.

Dropcho, E. J. (1996). Antiamphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis. *Ann. Neurol.* **39:** 659–667.

Duprey, P., and Lesens, C. (1994). Control of skeletal muscle-specific transcription: Involvement of paired homeodomain and MADS domain transcription factors. *Int. J. Dev. Biol.* **38:** 591–604.

Edmondson, D. G., Lyons, G. E., Martin, J. F., and Olson, E. N. (1994). Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120:** 1251–1263.

Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G. C. Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. *Oncogene,* in press.

Farrance, I. K., Mar, J. H., and Ordahl, C. P. (1992). M-CAT binding factor is related to the SV40 enhancer binding factor, TEF-1. *J. Biol. Chem.* **267:** 17234–17240.

Gorbea, C. M., Marchand, P., Jiang, W., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Bond, J. S. (1993). Cloning, expression, and chromosomal localization of the mouse meprin β subunit. *J. Biol. Chem.* **268:** 21035–21043.

Ishikawa, H., Silos, S. A., Tamai, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Uitto, J. (1994). cDNA cloning and chromosomal assignment of the mouse gene for desmoglein 3 (*Dsg3*), the pemphigus vulgaris antigen. *Mamm. Genome* **5:** 803–804.

Jenkins, N. A., Copeland, N. G., Taylor, B. A., and Lee, B. K. (1982). Organization, distribution and stability of endogenous ecotropic murine leukemia virus DNA sequences in chromosomes of *Mus musculus*. *J. Virol.* **43:** 26–36.

Kadlec, L., and Pendergast, A.-M. (1997). The amphiphysin-like protein 1 (ALP1) interacts functionally with the cABL tyrosine kinase

and may play a role in cytoskeletal regulation. *Proc. Natl. Acad. Sci. USA* **94:** 12390–12395.

Lyons, G. E., Micales, B. K., Schwarz, J., Martin, J. F., and Olson, E. N. (1995). Expression of mef2 genes in the mouse central nervous system suggests a role in neuronal maturation. *J. Neurosci.* **15:** 5727–5738.

Manly, K. F. (1993). A Macintosh program for storage and analysis of experimental genetic mapping data. *Mamm. Genome* **4:** 303–313.

Mayo, M. W., Wang, C. Y., Cogswell, P. C., Rogers-Graham, K. S., Lowe, S. W., Der, C. J., and Baldwin, A. S. (1997). Requirement of NF-kappaB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **278:** 1812–1815.

Molkentin, J. D., and Olson, E. N. (1996). Defining the regulatory networks for muscle development. *Curr. Opin. Genet. Dev.* **6:** 445–453.

Negorev, D., Reithman, H., Wechsler-Reya, R., Sakamuro, D., Prendergast, G. C., and Simon, D. (1996). The Bin1 gene localizes to human chromsome 2q1.4 by PCR analysis of somatic cell hybrids and fluorescence *in situ* hybridization. *Genomics* **33:** 329–331.

Olson, E. N., Perry, M., and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors. *Dev. Biol.* **172:** 2–14.

Ornatsky, O. I., Andreucci, J. J., and McDermott, J. C. (1997). A dominant-negative form of transcription factor MEF2 inhibits myogenesis. *J. Biol. Chem.* **272:** 33271–33278.

Owen, D. J., Wigge, P., Vallis, Y., Moore, J. D. A., Evans, P. R., and McMahon, H. T. (1998). Crystal structure of the amphiphysin-2 SH3 domain and its role in the prevention of dynamin ring formation. *EMBO J.* **17:** 5273–5285.

Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997). Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem.* **272:** 16700–16706.

Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M., and Baldwin, A. S. (1998). A requirement for NF-κB activation in Bcr-Abl-mediated transformation. *Genes Dev.* **12:** 968–981.

Sakamuro, D., Elliott, K., Wechsler-Reya, R., and Prendergast, G. C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nat. Genet.* **14:** 69–77.

Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996). Cloning of ligand targets: Systematic isolation of SH3 domain-containing proteins. *Nat. Biotech.* **14:** 741–744.

Tsutsui, K., Maeda, Y., Tsutsui, K., Seki, S., and Tokunaga, A. (1997). cDNA cloning of a novel amphiphysin isoform and tissue-specific expression of its multiple splice variants. *Biochem. Biophys. Res. Commun.* **236:** 178–183.

Walsh, K., and Perlman, H. (1997). Cell cycle exit upon myogenic differentiation. *Curr. Opin. Genet. Devel.* **7:** 597–602.

Wasserman, W. W., and Fickett, J. W. (1998). Identification of regulatory regions which confer muscle-specific gene expression. *J. Mol. Biol.* **278:** 167–181.

Wechsler-Reya, R., Elliott, K., Herlyn, M., and Prendergast, G. C. (1997a). The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. *Canc. Res.* **57:** 3258–3263.

Wechsler-Reya, R., Elliott, K., and Prendergast, G. C. (1998). A role for the putative tumor suppressor Bin1 in muscle cell differentiation. *Mol. Cell. Biol.* **18:** 566–575.

Wechsler-Reya, R., Sakamuro, D., Zhang, J., Duhadaway, J., and Prendergast, G. C. (1997b). Structural analysis of the human BIN1 gene: Evidence for tissue-specific transcriptional regulation and alternate RNA splicing. *J. Biol. Chem.* **272:** 31453–31458.

Wigge, P., Kohler, K., Vallis, Y., Doyle, C. A., Owen, D., Hunt, S. P., and McMahon, H. T. (1997a). Amphiphysin heterodimers: Potential role in clathrin-mediated endocytosis. *Mol. Biol. Cell* **8:** 2003–2015.

Wigge, P., Vallis, Y., and McMahon, H. T. (1997b). Inhibiton of receptor-mediated endoctosis by the amphiphysin SH3 domain. *Curr. Biol.* **7:** 554–560.

Oncogene (1999) 18, 2942–2954

http://www.stockton-press.co.uk/onc

New Myc-interacting proteins: a second Myc network emerges

Daitoku Sakamuro*,[1] and George C Prendergast[1]

[1]*The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania, PA 19104-4268, USA*

Despite its intensive investigation for almost two decades, c-Myc remains a fascinating and enigmatic subject. A large and compelling body of evidence indicates that c-Myc is a transcription factor with central roles in the regulation of cell proliferation, differentiation, and apoptosis, but its exact function has remained elusive. In this review we survey recent advances in the identification and analysis of c-Myc-binding proteins, which suggest insights into the transcriptional roles of c-Myc but which also extend the existing functional paradigms. The C-terminal domain (CTD) of c-Myc mediates interaction with Max and physiological recognition of DNA target sequences, events needed for all biological actions. Recently described interactions between the CTD and other cellular proteins, including YY-1, AP-2, BRCA-1, TFII-I, and Miz-1, suggest levels of regulatory complexity beyond Max in controlling DNA recognition by c-Myc. The N-terminal domain (NTD), which includes the evolutionarily conserved and functionally crucial Myc Box sequences (MB1 and MB2), contains the transcription activation domain (TAD) of c-Myc as well as regions required for transcriptional repression, cell cycle regulation, transformation, and apoptosis. In addition to interaction with the retinoblastoma family protein p107, the NTD has been shown to interact with α-tubulin and the novel adaptor proteins Bin1, MM-1, Pam, TRRAP, and AMY-1. The structure of these proteins and their effects on c-Myc actions suggest links to the transcriptional regulatory machinery as well as to cell cycle regulation, chromatin modeling, and apoptosis. Investigations of this emerging NTD-based network may reveal how c-Myc is regulated and how it affects cell fate, as well as providing tools to distinguish the physiological roles of various Myc target genes.

Keywords: neoplastic transformation; apoptosis; transcription; oncogene; tumor suppressor; proliferation

The Myc family of nuclear oncoproteins are key cell growth regulators that are oncogenically activated in a large fraction of human malignancies (reviewed in Cole, 1986; Kelly and Siebenlist, 1986; Spencer and Groudine, 1991). Most investigations have focused on the product of the c-Myc gene, which is widely expressed, but related Myc family genes that are less broadly expressed are also activated in malignancy (for example, N-Myc, which is activated in neuroblastoma, and L-Myc, which is activated in lung carcinoma; De Pinho *et al.*, 1991). c-Myc can be functionally deregulated by both genetic or epigenetic mechanisms, for example, by chromosomal translocation or by constitutive expression due to activation of upstream-acting growth factor receptors, respectively. Many studies have documented c-Myc overexpression in cancer. However, because deregulation rather than overexpression of Myc is sufficient for oncogenic activation, the involvement of Myc in cancer may be much broader than indicated by these studies. c-Myc is crucial for cell proliferation in normal and neoplastic settings. Following mitogenic stimulation of normal quiescent cells, c-Myc is rapidly induced and remains elevated, suggesting that it is required for continuous cell growth. Although its exact role is not entirely clear, c-Myc appears to be needed at several points during the cell cycle (Evan and Littlewood, 1993). Induction of c-Myc is sufficient to drive quiescent cells into the cell cycle (Eilers *et al.*, 1989), while inhibition of Myc can block mitogenic signals and facilitate cell differentiation (Hanson *et al.*, 1994; Heikkila *et al.*, 1987; Holt *et al.*, 1988; Sawyers *et al.*, 1992; Sklar *et al.*, 1991). Associated with its ability to drive proliferation is an equally potent ability to drive apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992). However, this feature is latent and only revealed in normal cells if c-Myc expression is uncoupled from survival signals mediated by cytokine and adhesion receptors (see Prendergast, this issue).

The mechanisms through which c-Myc mediates its diverse effects on cell fate are unknown. A broad body of work argues convincingly that c-Myc is a transcription factor which activates and represses different target genes (Amati *et al.*, 1998; Bouchard *et al.*, 1998; Dang, 1999; Facchini and Penn, 1998; Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Prendergast, 1997; Steiner *et al.*, 1996). However, there are a large number of studies which suggest that c-Myc has unique aspects beyond those associated with 'classical' transcription factors (Lemaitre *et al.*, 1996; Prendergast, 1997) and the identification of cellular factors which interact with c-Myc in cells would provide deeper insight into other possible function(s). The C-terminal domain (CTD) of c-Myc mediates DNA binding to physiological target genes through a heterodimer complex with basic region/helix–loop–helix/'leucine zipper' (b/HLH/Z) protein Max. The N-terminal domain (NTD) includes the transcription activation domain (TAD). Two short segments in the NTD termed Myc boxes 1 and 2 (MB1 and MB2) are conserved in all Myc family proteins and are crucial for all biological activities. The NTD also mediates transcriptional repression (Li *et al.*, 1994; Philipp *et al.*, 1994). Both the NTD and

*Correspondence: D Sakamuro

CTD are crucial for all biological activities whereas the central portion of c-Myc is dispensable. In recent years, barriers to the identification of cellular proteins which interact with c-Myc have begun to give way, providing new avenues for investigation of c-Myc function. New CTD-interacting proteins reinforce the role of this region in mediating specific DNA binding and suggest novel levels at which DNA binding is regulated. New NTD-binding proteins suggest links to the transcriptional regulatory machinery, including the anticipated link to complexes that contain histone acetylases, but also hint at novel connections to cell cycle regulation, chromatin remodeling, and apoptosis. Table 1 presents a list of the c-Myc-interacting proteins surveyed in this review along with their potential roles.

CTD-interacting proteins

The essential CTD spans aa 360–437 in c-Myc and is comprised of the b/HLH/Z domain. In addition to Max, the proteins Nmi, YY-1, AP-2, TFII-I, BRCA1, and Miz-1 have each been implicated in interactions with this region. All these proteins have been linked to transcriptional regulation, most strongly in the cases of YY-1, AP-2, and TFII-I. Investigations of the interactions between c-Myc and these proteins have strengthened the notion that the function of the CTD is to control the access of the NTD to particular genetic loci. However, the results of several investigations have also prompted the idea that the association of c-Myc may have a reciprocal regulatory effect on the CTD-binding factors, especially in the cases of YY-1 and TFII-I. In this sense, some of the recent work introduces the idea that the CTD may also act as a domain that regulates the activity of other transcription factors, by sequestering them or preventing their interaction with other factors (such as, for example, the interaction of YY-1 with TBP or TFII-B, or the interaction of TFII-I with TBP.

Table 1 Effects on Myc function. Myc-interacting proteins surveyed in this review are summarized with regard to their reported effects on c-Myc function

Interacting protein	*Function*[a]		
	Transactivation	*Cell transformation*	*Apoptosis*
NTD-binding			
p107	–	–	ND
BIN1	–	–	+
TRRAP	ND[b]	+	–[c]
MM-1	–	–[d]	ND
AMY-1	+	ND	ND
Pam	ND	ND	ND
α-Turbulin	ND	ND	ND
CTD-binding			
Max	+	+	+
YY1	–	–	ND
AP2	–	ND	ND
TFII-I	–	ND	ND
BRCA1	–	–	ND
Miz-1	–[e]	ND	ND

[a]–, inhibitory effect; +, positive or necessary effect; ND, not determined. [b]TRRAP is a component of the transcriptional adaptor complex SAGA which contains histone acetyltransferases (HATs). It has yet to be explicitly shown that HAT activity is required for Myc to drive proliferation. [c]M Cole, pers. comm. [d]H Ariga, pers. comm. [e]transactivation by Miz-1 is inhibited by interaction with the Myc CTD (see text)

Max

The identification of Max, a physiological b/HLH/Z partner protein for c-Myc, was a milestone that provided the basic insight into how c-Myc recognizes DNA (reviewed in Blackwood *et al.*, 1992b; Henriksson and Lüscher, 1996; Kato and Dang, 1992; Meichle *et al.*, 1992; Prendergast and Ziff, 1992). The function of Max is to control the access of Myc proteins to their physiological DNA recognition sites. Max is essentially comprised of a b/HLH/Z domain and a C-terminal nuclear localization sequence. X-ray crystallographic analysis of the b/HLH/Z from this simple protein provided the first glimpse of the structure of the HLH domain (Ferre-D'Amare *et al.*, 1993). Heterodimerization with Max is necessary for c-Myc to mediate proliferation, transformation, and apoptosis (Amati *et al.*, 1993a,b; Mukherjee *et al.*, 1992; Prendergast *et al.*, 1992). Homodimerization of Max occurs *in vitro* but it is unclear if homodimerization is meaningful *in vivo* (Yin *et al.*, 1998). The DNA binding capacity of Max is subject to regulation by casein kinase II-mediated phosphorylation proximal to the basic region and by alternate splicing (Arsura *et al.*, 1995; Berberich *et al.*, 1992; Bousset *et al.*, 1993; Mäkelä *et al.*, 1992; Prochownik and Van Antwerp, 1993; Vastrik *et al.*, 1995). Max is a long-lived and constitutively expressed protein whose levels are upregulated several-fold by serum growth factors in all cells examined except certain 3T3 cells (Berberich *et al.*, 1992; Blackwood *et al.*, 1992a; Martel *et al.*, 1995; Prendergast *et al.*, 1991; Shibuya *et al.*, 1992; Wagner *et al.*, 1992). Examples of Max deletion have been seen in PC12 pheochromocytoma cells (Hopewell and Ziff, 1995), suggesting that other Myc partner proteins exist. Although the human Max gene lies at chromosome 14q23 where abnormalities occur in some cancers (Wagner *et al.*, 1992), alterations of Max do not appear to occur in malignant cells.

Max also interacts with the Mad family of b/HLH/Z proteins which are implicated in transcriptional repression, cell growth inhibition, and differentiation (Amati and Land, 1994; Foley *et al.*, 1998; Hurlin *et al.*, 1994; Schreiber-Agus and DePinho, 1998). Mad/Max heterodimers recognize Myc/Max E box sequences and repress transcription via Mad-dependent interactions with the corepressor protein mSin3 (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995), which recruits histone deacetylases that remodel chromatin and suppress transcription (Hassig *et al.*, 1997; Laherty *et al.*, 1997). Max and Mad arose earlier in evolution earlier than Myc. For example, homologs of Max and Mad but not of Myc can be found in *C. elegans* that function similarly to their human counterparts (Yuan *et al.*, 1998), whereas Myc has been found only as far down the evolutionary tree as *Drosophila* (Gallant *et al.*, 1996). Thus, Max stands at a crossroads between Myc and Mad proteins whose actions oppose each other in proliferation and differentiation (Chin *et al.*, 1995). Max also heterodimerizes with the b/HLH/Z protein Rox/Mnt, which is also implicated in binding to Myc/Max and Mad/

Max DNA binding sites, differentiation, and transcriptional repression via an mSin3-dependent mechanism (Hurlin *et al.*, 1997; Meroni *et al.*, 1997). Max switches from Myc to Mad and probably Mnt during differentiation (Ayer and Eisenman, 1993; Cultraro *et al.*, 1997; Hurlin *et al.*, 1997; Larsson *et al.*, 1997; Meroni *et al.*, 1997), although Max may not be an obligate partner in all Mad complexes during differentiation (Gupta *et al.*, 1998; Ryan and Birnie, 1997a). Recent investigations of Max are considered further by Luscher and Larsson in this issue.

Nmi

Nmi was identified in a yeast two hybrid screen for proteins that could interact with the N-Myc HLH/Z region (Bao and Zervos, 1996). While the function of Nmi is unknown its C-terminus is similar to an interferon-induced leucine zipper protein, IFP 35. Nmi binds to both c-Myc and N-myc in cells and to other transcription factors in yeast. It is expressed at low levels in all fetal and adult human tissues tested, except brain, and high levels are seen in certain myeloid leukemias which also express high levels of c-*myc* (Bao and Zervos, 1996). The *nmi* gene is located at human chromosome 22q13.3, a region which is reportedly translocated in some human leukemias. A recent report in which Nmi was identified as a STAT-binding protein suggests a more universal role of Nmi in transcriptional regulation (Zhu *et al.*, 1999). Nmi does not have any intrinsic transactivation domain but binds to all STAT proteins except STAT2 and augments STAT-activated transcription by recruiting the coactivator proteins CBP/p300. It will be of interest, therefore, to determine if Nmi plays any role in recruiting coactivator proteins to N-Myc and c-Myc, although the basis for biochemical association between Nmi and CBP/p300 in the context of STATs is unclear at this point.

YY-1

The C-terminal half of c-Myc including the central domain and CTD was identified in a yeast two hybrid screen for proteins that could interact with the transcription factor Yin-Yang-1 (YY-1) (Shrivastava *et al.*, 1993). YY-1 regulates the transcription of many genes, including possibly c-*myc* itself (Riggs *et al.*, 1993). Depending on the context, YY-1 can serve as a repressor, activator, or initiator of transcription. YY-1 associates with c-Myc but not Max and the interaction of YY-1 with c-Myc precludes Max binding (Shrivastava *et al.*, 1993). Additional binding determinants for YY-1 appear to be located in the biologically dispensable central region of c-Myc. In transient cotransfection assays, c-Myc inhibited both the repressor and the activator functions of YY-1, suggesting that one way c-Myc may act is by modulating the transcriptional activities of YY-1. A subsequent study provided evidence of *in vivo* interaction and argued that association occurred preferentially in cells expressing higher levels of c-Myc (Shrivastava *et al.*, 1996). In addition, the results of more detailed binding experiments implied that c-Myc inhibited YY-1 activity not blocking its binding to DNA but by competitively inferring with its ability to interact with the basal transcription factors TBP and/or TFII-B (Shrivastava *et al.*, 1996). Consistent with physiological interaction, YY-1 was shown recently to inhibit Myc/Ras cotransformation (Austen *et al.*, 1998). However, this effect depended on DNA binding by YY-1 but not on interaction with c-Myc. Therefore, YY-1 may also act indirectly to affect c-Myc activity. Indirect regulation of c-Myc by YY-1 was further supported by the finding that although YY-1 did not bind to the c-Myc (TAD) *in vitro* it was able to inhibit transactivation by a Gal4-c-MycTAD chimeric protein in transient activation assays (Austen *et al.*, 1998). The link between YY-1 and c-Myc is considered further in the review by Luscher and Larsson in this issue.

AP-2

One study has implied direct and indirect interactions between the c-Myc CTD and the transcription factor AP-2 (Gaubatz *et al.*, 1995). This link was revealed by the presence of overlapping binding sites for AP-2 and c-Myc in the Myc target genes prothymosin-α and ornithine decarboxylase (ODC). AP-2 can compete with c-Myc/Max heterodimers for binding to DNA and thereby inhibit their ability to activate transcription in an indirect manner. However, AP-2 can also interact with c-Myc directly via the CTD of AP-2 and the b/HLH/Z domain of Myc. In contrast, neither Max nor Mad bind to AP-2. The interaction of AP-2 with c-Myc does not preclude association with Max, but it impairs DNA binding of the Myc/Max complex and inhibits transactivation by Myc in the absence of an overlapping AP-2 binding site. Thus, AP-2 acts as a negative regulator of transactivation by Myc. This mechanism of action may be important in cells where Mad is absent or poorly expressed during development, when expression of AP-2 and Mad is nonoverlapping (Gaubatz *et al.*, 1995; Morriss-Kay, 1996). While a role for AP-2 in the regulation of proliferation by c-Myc has not been reported, AP-2 expression is reported to inhibit apoptosis by c-Myc (Moser *et al.*, 1997). Thus, AP-2 may block binding of c-Myc/Max to target genes with critical roles in apoptosis, such as ODC (Packham and Cleveland, 1994).

TFII-I

Interactions between c-Myc and the transcription factor TFII-I appear to be involved in transcriptional repression by c-Myc at initiator (Inr) elements which overlap the cap site in many promoters (Li *et al.*, 1994; Roy *et al.*, 1993a,b). TFII-I has been shown to bind independently to two distinct promoter elements, the pyrimidine-rich Inr site as well as E-box sites which can be regulated by c-Myc or the distantly related b/HLH protein upstream stimulatory factor 1 (USF1) (Roy *et al.*, 1997). The primary structure of TFII-I reveals novel features that include six directly repeated 90 residue motifs that each possess a potential helix–loop/span-helix homology. These unique structural features suggest that TFII-I may have the capacity for multiple protein-protein and, potentially, multiple protein-DNA interactions. Consistent with this likelihood TFII-I has shown recently

to stabilize and stimulate the transactivation activity of ternary complexes of the serum response factor (SRF) and the homeobox-containing protein Phox1 (Grueneberg *et al.*, 1997). *In vitro* binding studies suggest that TFII-I and USF1 act synergistically to activate transcription through both Inr and the E-box elements of the adenovirus major late promoter (Roy *et al.*, 1997), which is also a target site for c-Myc (Prendergast *et al.*, 1991; Prendergast and Ziff, 1991). c-Myc interacts with TFII-I and binds cooperatively at both Inr and upstream E-box promoter elements in a manner similar to USF1 (Roy *et al.*, 1993a). However, unlike the case with USF1, c-Myc interactions at the Inr lead to an inhibition of transcription initiation by TFII-I, apparently by precluding the ability of the latter to contact TBP (Roy *et al.*, 1993a,b). This inhibition is selective for a TFII-I-dependent (as opposed to TFIIA-dependent) initiation pathway and correlates with the prevention of complex formation between TBP, TFII-I, and the promoter (Roy *et al.*, 1993a). Not all genes repressed by c-Myc have Inr sequences, but for those that do the ability of c-Myc to interfere with TFII-I seems the mostly likely mechanism of action.

BRCA-1

Using the central region of the familial breast tumor suppressor BRCA1 as bait in a two hybrid screen the c-Myc CTD was identified as a BRCA1-interacting protein (Wang *et al.*, 1998). BRCA1 associated with c-Myc in *in vitro* binding assays and association between endogenous proteins was documented in mammalian cells. An intact HLH region was required in c-Myc for efficient association with BRCA1 and specificity was argued by the inability of Max to interact similarly with BRCA1. Consistent with association in cells, overexpression of BRCA1 selectively repressed c-Myc-mediated transactivation and inhibited Ras cotransformation of embryonic fibroblasts by c-Myc but not by SV40 T antigen. Thus, BRCA1 may act in part by regulating the oncogenic potential of c-Myc, providing a molecular explanation for some of the biological effects of the BRCA1 gene product (Wang *et al.*, 1998). A role in governing apoptosis by c-Myc might be entertained since the lethality of BRCA1 nullizygous mice can be rescued in a p53 or $p21^{WAF1}$ nullizygous background (Hakem *et al.*, 1997) and p53 elevation can sensitize cells to the cytotoxic effects of c-Myc (Hermeking and Eick, 1994; Wagner *et al.*, 1994). Another possibility is suggested by studies indicating a role for BRCA1 in transcription-coupled DNA repair (Bertwistle and Ashworth, 1998; Gowen *et al.*, 1998; Scully *et al.*, 1997b). Activation of BRCA1 following DNA damage (Scully *et al.*, 1997a) might promote cell cycle arrest and/or repair in part by inhibiting c-Myc/Max interaction or DNA binding. Further investigation is needed to determine how BRCA1 interaction might regulate the physiological functions of c-Myc.

Miz-1

The zinc finger protein Miz-1 was identified in a two hybrid screen for proteins that could interact with the c-Myc CTD (Peukert *et al.*, 1997). Miz-1 has in addition to its zinc fingers a POZ domain that functions as a transcriptional repression function. Domain mapping revealed that interaction required the HLH domain of c-Myc and an amphipathic helix in the C-terminus of Miz-1. Neither Max nor USF interacted with this protein. Miz-1 binds initiator elements at the adenovirus major late and cyclin D1 promoters, each of which are genetic targets of c-Myc (Philipp *et al.*, 1994; Prendergast and Ziff, 1991), and activates transcription from both promoters. Miz-1 has a potent growth arrest function. Expression of c-Myc inhibits transactivation and overcomes Miz-1-induced growth arrest in manner dependent on c-Myc/Miz-1 association and on the integrity of the POZ domain, suggesting that binding to c-Myc activates a latent repressive function of this domain. Notably, Miz-1 is a cytosolic protein whose association with c-Myc mediates nuclear localization. Fusion of a nuclear localization signal to Miz-1 induces nuclear transport and impairs the ability of c-Myc to overcome Miz-1-dependent transcriptional activation and growth arrest. Although the transcriptional targets of Miz-1 are undefined, a possible role for Miz-1 has been recently reported as a stabilizer of Myc against ubiquitin-mediated proteolysis (Salghetti *et al.*, 1999).

NTD-interacting proteins

The mysteries concerning the exact function(s) of Myc are lodged in its NTD. This part of c-Myc spans ~150 N-terminal residues and constitutes the second biologically essential region of c-Myc in addition to the CTD. c-Myc is unusual in that its messenger RNA encodes two polypeptide species, one of which is derived from a non-AUG translation initiation site that results in a short N-terminal extension relative to the shorter AUG-initiated species (Hann *et al.*, 1988). Interestingly, the longer form (which is disrupted in certain cancers (Hann *et al.*, 1988) has different DNA binding properties that allow it to recognize and activate transcription from a non-E-box site (Hann *et al.*, 1994), the antisense sequence of which was actually originally identified as a Myc binding site in a study assessing a putative but controversial role for c-Myc in DNA replication (Iguchi-Ariga *et al.*, 1987). How the N-terminal extension on the NTD influences the DNA binding properties of c-Myc is not understood.

The NTD includes two short sequence motifs of <20 aa that are highly conserved among Myc family proteins, termed the Myc-homology Box 1 (MB1) and Box 2 (MB2), which are located at approximately aa 42–65 and 130–143 in human c-Myc, respectively (aa below also refer to human c-Myc; see Figure 1 for reference). MB1 is the major site of *in vivo* phosphorylation events that regulate transcription and transformation and it is a hotspot for mutation in cancer (reviewed in Henriksson and Lüscher, 1996; Prendergast, 1997). In particular, T58 and S62 within the MB1 region are phosphorylated in a cell cycle-dependent manner, possibly by certain CDKs, MAPK, and/or GSK-3β (Gupta *et al.*, 1993; Haas *et al.*, 1997;

Lutterbach and Hann, 1994; Seth *et al.*, 1993; Sterner *et al.*, 1996). S62 appears to be play a positive role since its mutation drastically reduces transforming activity (Henriksson *et al.*, 1993; Lutterbach and Hann, 1994). In contrast, T58 appears to be a negative regulatory site which it is frequently mutated in Burkitt's lymphomas and retroviral *myc* genes (Bhatia *et al.*, 1993; Henriksson *et al.*, 1993; Lutterbach and Hann, 1994; Papas and Lautenberger, 1985; Showe *et al.*, 1985; Yano *et al.*, 1993). Interestingly, T58 is also a site for glycosylation, although the functional meaning of this event is unclear (Chou *et al.*, 1995). MB2 encodes the most hydrophobic part of Myc, so it is likely to be either a core organizer for the NTD fold or an interaction motif that is not constitutively exposed to a hydrophilic environment. A combination of mutagenesis and structure-function analysis has identified two TADs in the NTD, one of which is located immediately upstream of MB1 (aa 1–41) and the second of which is located upstream and overlapping with MB2 (aa 103–144) (Prendergast, 1997). MB1 and sequences immediately downstream (aa 42–91) have little activation or repression activity, suggesting that MB1 is the key part of a regulatory or effector region which controls or is controlled by adjacent regions. Interestingly, sequences in the MB2-overlapping TAD are conserved in particular Myc family proteins in evolution (e.g. in c-Myc or N-Myc), but they diverge between different family members (approximately aa 100–130). This suggests that some nonredundant function(s) of the Myc family is encoded by this region. An important repression element lies between MB1 and the MB2 TAD (aa 92–106) and a second overlaps MB2 itself (Prendergast, 1997). Both MB1 and MB2 are important for Myc biology but MB2 is completely essential (Brough *et al.*, 1995; Evan *et al.*, 1992; Li *et al.*, 1994; Stone *et al.*, 1987). In recent years, as attention has shifted to the NTD in functional analysis of Myc proteins, attention has been focused on MB1 and MB2 as crucial elements for protein-protein interaction because of their key biological roles.

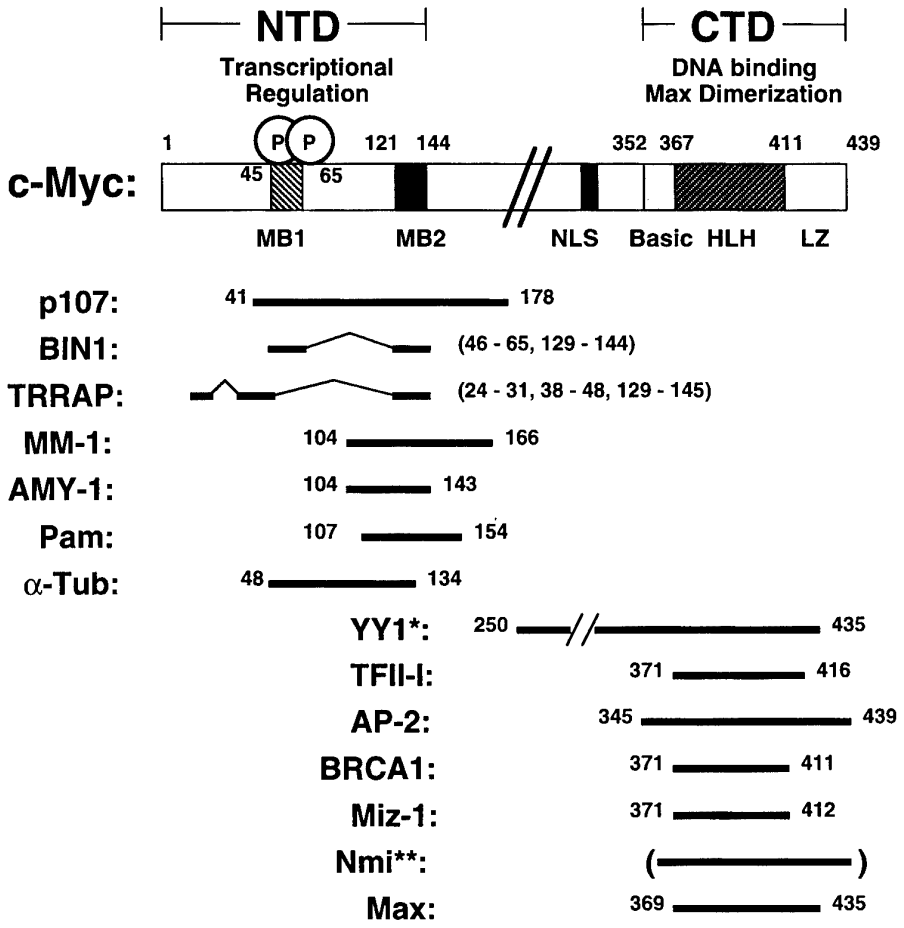


Figure 1 c-Myc structure and regions implicated in protein-protein interaction. The structure of human c-Myc structure is depicted. Functionally critical phosphorylation sites at S62 and T58 in MB1 are shown by circled P. Black bars depict the extent of regions implicated in interactions with the Myc-binding proteins listed. *YY-1 may affect c-Myc indirectly as well as possibly directly; **N-Myc CTD was used as bait to identify Nmi although c-Myc and N-Myc both interact (see text). NTD, N-terminal domain; CTD, C-terminal domain; MB1 and MB2, Myc boxes; NLS, nuclear localization signal; HLH, helix–loop–helix fold; LZ, leucine zipper

Two first proteins to be identified as candidates for NTD interaction were the transcription factor TBP and the retinoblastoma (Rb) family protein p107 (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994; Hateboer *et al.*, 1993). More recently, α-tubulin and the adaptor proteins Bin1, TRRAP, Pam, MM-1, and AMY-1 have been identified as NTD-binding proteins. The identification and analysis of NTD-interacting proteins had been impeded by problematic biochemical aspects of c-Myc, which is difficult to express recombinantly and is so recalcitrant to isolation in its native form that its *in vivo* DNA binding properties were reliably documented only quite recently (Sommer *et al.*, 1998). Furthermore, the NTD is a strong transactivator in yeast, ruling out straightforward two hybrid screens. However, these barriers were eventually surmounted, resulting in the recent progress to identify NTD-binding proteins. One surprising theme that has emerged is that none of the proteins identified to date encode 'classical' transcriptional coactivator or corepressor proteins that might have been anticipated based on earlier progress on the Mad-Max system. However, given that Myc appeared later in evolution than Mad or Max, one might expect a baroque quality or at least lack of symmetry to the NTD network based on expectations from study of the CTD network. Indeed, the recent advances suggest that the NTD-based Myc network integrates transcriptional functions with other functions concerning cell cycle transit, chromatin modeling, and apoptosis or cell fate signaling.

p107

The identification of p107 as an NTD-interacting protein was cued by evidence of biological parallels in how c-Myc and adenovirus E1A caused cell transformation in collaboration with activated Ras (Land *et al.*, 1983; Ralston, 1991; Ruley, 1983). The interactions between E1A and the retinoblastoma protein (Rb) and p300/CBP that were required for cell transformation by that oncoprotein immediately suggested that c-Myc may have similar interactions. Early evidence that c-Myc and Rb could interact *in vitro* (Rustgi *et al.*, 1991) were not confirmed *in vivo*, although a later study provided evidence of indirect interaction between these proteins (Adnane and Robbins, 1995). Subsequent investigations of the Rb-related protein p107 revealed evidence of *in vivo* interaction with c-Myc and inhibition of its transactivating properties (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994). The so-called 'pocket' domain of p107 binds to the Myc NTD, requiring especially the MB2 region (Hoang *et al.*, 1995). p107 in complex with cyclin A/CDK2 kinase has been reported to inhibit cell cycle-dependent phosphorylation of MB1 *in vitro* (Hoang *et al.*, 1995), suggesting that c-Myc could be a downstream target of p107. In addition, p107 has been reported to mediate inhibition of c-Myc transactivation and transformation by p16INK4a (Haas *et al.*, 1997). Thus, the phosphorylation status of p107 which is achieved by the balance between p16INK4a and cyclin D/CDK4

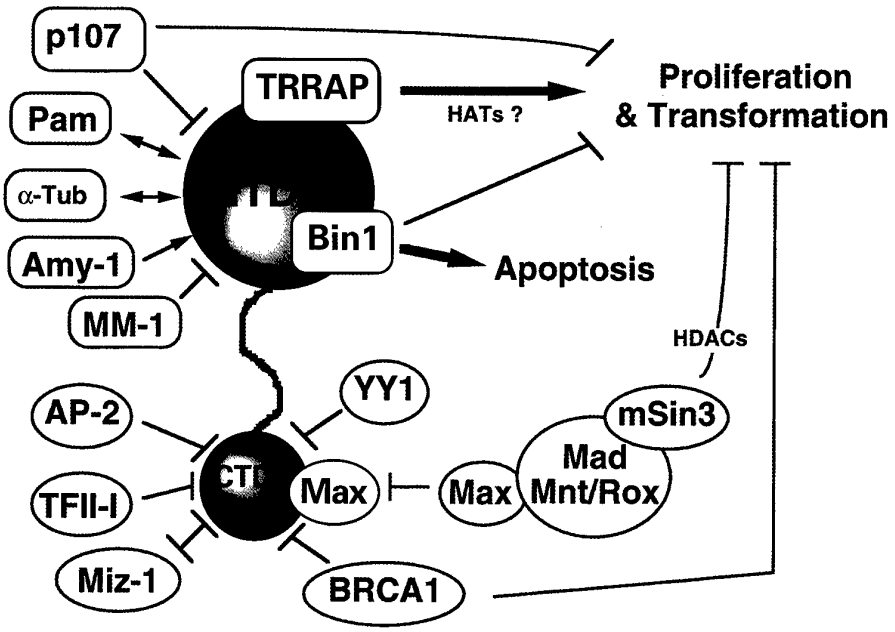


Figure 2 c-Myc lies at the intersection of CTD and NTD networks. The CTD includes the b/HLH/Z domain which interacts with the Max network and the other factors indicated. The NTD includes the Myc boxes MB1 and MB2 which mediate interactions with a second network of proteins implicated in transcriptional regulation, chromatin modeling, and apoptosis (cell fate regulation). Regulatory or effector proteins are listed on the left side of the figure and likely effector proteins listed on the right side. This figure proposes a dual signal model in which TRRAP and Bin1 mediate proliferative and apoptotic functions of c-Myc, respectively. c-Myc N-terminal domain; CTD, C-terminal domain; HATs, histone acetyltransferases; HDACs, histone deacetylases

actions may be a crucial regulatory determinant of c-Myc activity in the cell cycle (Amati *et al.*, 1998). However, the significance of c-Myc-p107 association in tumorigenesis is unclear, since there is conflicting evidence for the antitransforming activity of p107 in transformation (Haas *et al.*, 1997; Prendergast, 1997) as well as the ability of Burkitt's lymphoma-associated c-Myc mutants to escape inhibition by p107 in transcriptional activation assays (Hoang *et al.*, 1995; Smith-Sorensen *et al.*, 1996). However, although a consensus on the role of p107 in c-Myc mediated transactivation and cell proliferation has not fully emerged, p107 seems likely to be an important regulator of c-Myc function during the cell cycle. The fact that p107 is not mutated in cancer, like Rb, suggests that loss of this regulatory function is not crucial to deregulation of c-Myc in cancer cells, however. This negative evidence as well as the lack of a role for p107 in apoptosis, another function of c-Myc, suggested that other important NTD-binding proteins should exist to control and mediate the proliferative and proapoptotic properties of c-Myc.

TBP

In vitro binding between TBP and c-Myc has been described (Hateboer *et al.*, 1993) but *in vivo* confirmation of this result has not been reported. Still, TBP interaction may have some *in vivo* relevance, since several c-Myc-interacting proteins have been found to interact with TBP and c-Myc has been observed to influence these interactions *in vitro* and *in vivo*. The CTD-binding proteins YY-1 and TFII-I both interact tightly with TBP and through different domains with c-Myc (Roy *et al.*, 1993a,b; Shrivastava *et al.*, 1996), although TBP was reported to bind to c-Myc relatively weakly (Roy *et al.*, 1993a). Notably, c-Myc blocked transcriptionally productive interactions between TBP and YY-1 or TFII-I, consistent with the possibility that c-Myc contacts TBP in some way. Lastly, the NTD-binding protein Bin1 has been observed to bind to TBP *in vitro* via a region which is proximal to critical determinants for c-Myc binding (Elliott *et al.*, 1999). Further investigations are required to determine whether interaction between the c-Myc NTD and TBP are physiologically relevant or not.

α-tubulin

One line of investigation has led to the finding that c-Myc can associate with α-tubulin and polymerized microtubules in cells (Alexandrova *et al.*, 1995). This finding was prompted by observations that c-Myc shifts its localization to the cytosol in some cells as they exit the cell cycle (Vriz *et al.*, 1992), along with evidence that tubulin is responsible for the cytoplasm-to-nucleus translocation of certain proteins (Alexandrova *et al.*, 1995). c-Myc interacted with α-tubulin and microtubules in both *in vitro* binding experiments and *in vivo* immunoprecipitation and colocalization assays. The MB1-containing region from aa 48–134 was implicated in mediating the interaction. While the role of tubulin interaction is undetermined at this point, the results of this study are consistent with the hypothesis that microtubules may aid in subcellular trafficking of c-Myc that occurs under certain circumstances (Lemaitre *et al.*, 1996).

Bin1

MB1 was used in the two hybrid screen which identified the adaptor protein Bin1 (Sakamuro *et al.*, 1996). MB1 was chosen as an NTD bait because it was known to be displayed on the surface of native c-Myc protein (since *in vivo* phosphorylation occurred there); had a potentially important negative role in cancer (since it was mutated in malignant cells); and was nontransactivating in yeast. Bin1 is a nucleocytoplasmic adaptor protein which is in excess to c-Myc in cells. Although MB1 was used to identify Bin1 in yeast, the biochemical association of Bin1 with full-length c-Myc also requires MB2 (Sakamuro *et al.*, 1996). Consistent with dependence on MB1 and MB2, Bin1 functionally associates with c-Myc in cells and selectively inhibits its oncogenic and transactivation properties in a binding domain-dependent manner (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996). In Ras cotransformation assays Bin1 suppressed focus formation by c-Myc and deletion of the c-Myc binding domain (MBD) relieved suppression. Transformation by E1A, HPV E7, and mutant p53, but not SV40 T antigen were also susceptible to suppression by Bin1 via an MBD-independent mechanism. Instead, Bin1 suppression required different domains which are dispensable to bind Myc or suppress Myc transformation. Taken together, the results suggested that Bin1 could inhibit growth mediated either by deregulation of the c-Myc or the Rb/E2F systems (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996). Structure-function analysis identified a possible effector region, termed BAR-C, that was not involved in c-Myc binding but was indispensable to suppress Myc transformation and tumor cell growth (Elliott *et al.*, 1999). Bin1 selectively suppressed transactivation of artificial promoters responsive to Myc/Max or Gal4-Myc as well as natural Myc-regulated promoters, including those of the ODC and α-prothymosin (pT) genes. MBD was required for suppression of Myc activation of ODC not α-pT, suggesting that Bin1 may act by more than one mechanism (Elliott *et al.*, 1999). Consistent with this possibility, the MBD could associate with TBP *in vitro* and Gal4-Bin1 fusion proteins recruited a repression function unrelated to histone deacetylases in a manner independent of the MBD (Elliott *et al.*, 1999). A determination of the physiological role of Bin1 in transcription, if any, requires further investigation.

Bin1 has several features of a tumor suppressor. The human Bin1 gene (Wechsler-Reya *et al.*, 1997) is located at chromosome 2q14 (Negorev *et al.*, 1996), which lies within a hotspot for deletion in metastatic prostate cancers (Cher *et al.*, 1996). Loss of an adaptor protein that suppresses the oncogenic properties of c-Myc may be important in prostate cancer since c-Myc deregulation is among the most frequent events in this malignancy (Bova and Isaacs, 1996). A recent study provided evidence of loss of heterozygosity at the Bin1 locus in 40% of genomic DNAs

from matched samples of DNA from cases of normal and malignant prostate but not bladder tissue. Bin1 is missing or epigenetically altered in >50% primary breast and prostate tumors and cell lines and also in malignant melanoma (Ge *et al.*, manuscript submitted). Reintroduction of the wild-type gene inhibits the growth of tumor cell lines lacking endogenous Bin1 but not in cells expressing wild-type Bin1; growth inhibition is due to apoptosis (Sakamuro *et al.*, 1996; Ge *et al.*, manuscript submitted; Elliot *et al.*, manuscript in preparation). Similar to other tumor suppressors Bin1 is necessary for myoblast differentiation, where it acts at an early step before $p21^{WAF1}$ elevation (Mao *et al.*, 1999; Wechsler-Reya *et al.*, 1998). Interestingly, during differentiation of myoblasts, smooth muscle, and keratinocytes, Bin1 is relocalized from mainly nuclear sites to mainly cytosolic sites (Wechsler-Reya *et al.*, 1998; GC Prendergast, unpublished observations). Bin1 interaction with c-Myc in myoblasts might provide an explanation for recent results suggesting that c-Myc blocks differentiation in manner separate from its cell proliferative effects (Ryan and Birnie, 1997b), similar to E1A (Frisch, 1997).

Investigations to identify the basis for Bin1 action indicate that it has a necessary role in the mechanism by which c-Myc activates apoptosis (Sakamuro *et al.*, manuscript submitted). In primary chick fibroblasts, where c-Myc is sufficient to drive transformation or apoptosis, expression of antisense or dominant inhibitory Bin1 genes slightly enhanced cell proliferation and anchorage-independent growth but greatly reduced the susceptibility of cells to c-Myc-induced apoptosis elicited by serum deprival. A requirement for Bin1 interaction in apoptosis was implied by the finding that overexpression of the MBD, which dominantly interferes with the c-Myc-Bin1 interaction, rendered cells resistant to Myc-induced apoptosis (Sakamuro *et al.*, manuscript submitted). In a baby rat kidney (BRK) epithelial system where c-Myc can drive p53-independent apoptosis (Sakamuro *et al.*, 1995), inhibition of Bin1 promoted cell proliferation and stanched cell death. Notably, Bin1 inhibition masked the cytotoxic effects of Myc to a degree that was similar to Bcl-2 and that was sufficient to support cell outgrowth under suboptimal growth factor conditions. Overexpression of Bin1 did not kill IMR90 human diploid fibroblasts, but did kill tumor cells lacking endogenous Bin1 where c-Myc was deregulated. The antitransforming effects of Bin1 against c-Myc in Ras cotransformation assays may be based on switching the balance of Myc signaling to a proapoptotic effector, because overexpression of the MBD appears to both promote transformation and proliferation and reduce apoptosis in cells where c-Myc is overexpressed. Taken together, these results strongly support the 'dual signal' model for Myc function and suggest that Bin1 is an adaptor-effector that mediates death or death sensitization signals from c-Myc (see Prendergast, this issue).

Bin1 is in excess to c-Myc in cells and also has c-Myc-independent roles in cell regulation modulated by alternate splicing (Weschler-Reya *et al.*, 1997, 1998; Elliott *et al.*, unpublished observations). Thus, like most adaptor proteins, Bin1 probably participates in diverse interactions in the cell. Existing evidence suggests some role for Bin1 in coordinating cell fate decisions that are made when cells exit the cell cycle (e.g. arrest in G0, commit to differentiate, undergo apoptosis, etc.). For example, as shown above, if cells cannot exit the cell cycle due to c-Myc deregulation, then Bin1 is necessary to mediate an abortive apoptotic signal. Alternately, if c-Myc is downregulated appropriately and as a result cells are able to exit the cell cycle, then Bin1 appears to promote arrest and differentiation (Mao *et al.*, 1999; Wechsler-Reya *et al.*, 1998). Additional information supports a complex role in cells. Bin1 is subjected to extensive alternate splicing, especially in neurons (Butler *et al.*, 1997; Ramjaun and McPherson, 1998; Ramjaun *et al.*, 1997; Tsutsui *et al.*, 1997; Wechsler-Reya *et al.*, 1997), and it is localized to the cytosol as well as the nucleus in certain cells (Butler *et al.*, 1997; Kadlec and Pendergast, 1997; Wechsler-Reya *et al.*, 1998). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein and paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.*, 1994; Dropcho, 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.*, 1993; Crouzet *et al.*, 1991). Amphiphysin and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin isoform, have been implicated in receptor-mediated endocytosis (David *et al.*, 1996; Owen *et al.*, 1998; Wigge *et al.*, 1997). RVS167 and RVS161 have been implicated in endocytosis and karyogamy (Brizzio *et al.*, 1998; Munn *et al.*, 1995). However, nonneuronal splice forms of Bin1 are unlikely to be involved in endocytosis, because only neuronal splice forms include exons which encode clathrin-binding determinants needed for localization to endocytotic vesicles (Ramjaun and McPherson, 1998). It is hypothesized that the endocytosis connection in neurons reflects the link between survival and the achievement of a differentiated and synaptically active state in those cells, which would be associated with neurotransmitter release and hence membrane trafficking. Recently, the nuclear tyrosine kinase c-Abl was shown to associate with but not to phosphorylate Bin1 in cells (Kadlec and Pendergast, 1997). Association of c-Abl with Bin1 is mediated by its SH3 domain, which is dispensable for association with c-Myc (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996). How c-Abl and Bin1 influences each other's actions in cell fate decisions remains to be determined. In summary, Bin1 is a nucleocytoplasmic cell fate adaptor with an important role in mediating apoptosis and possibly other cell fate decisions influenced by c-Myc. Continued investigations of Bin1 may open up new vistas on Myc function.

MM-1

MM-1 (Myc Modulator-1) was identified in a two hybrid screen for proteins that could interact with the entire c-Myc protein (Mori *et al.*, 1998). MM-1 is a nucleocytoplasmic protein that is ubiquitously expressed in normal tissues. Evidence that MM-1 interacted with c-Myc derived from extensive yeast and mammalian two-hybrid assays and *in vitro* GST binding assays. Deletion analyses showed that most of

the MM-1 protein and a segment of the c-Myc NTD encompassing MB2 (aa 104–166) were necessary for protein-protein association. It was not determined whether the a leucine zipper in the N-terminus of MM-1 was required for formation of homo- or hetero-dimers via this motif. MM-1 suppressed the transcriptional activation by c-Myc in a dose-dependent manner, consistent with functional interaction in cells. Inhibitory effects of MM-1 on c-Myc-dependent cell proliferation have also been observed (H Ariga, personal communication). It should be noted that MM-1 binds to Myc NTD via MB2 region that is critical for Myc transformation. Thus, MM-1 may compete for binding to c-Myc with TRRAP, a second recently identified MB2-binding protein that promotes Myc transformation. Interestingly, MM-1 may be a human homolog of GIM5, a subunit in a complex which promotes the formation of functional α-tubulin and γ-tubulin. This link reinforces the idea that c-Myc may associate with tubulin toward some functional end in cells. Further analysis concerning the effect of MM-1 on the transforming and apoptotic properties of c-Myc and of the structure and expression of the human *MM-1* gene in primary tumor cells would advance the significance of MM-1 to Myc function, especially in cancer.

TRRAP

One of the pressing issues regarding Myc function is how transcriptional activation and repression is mediated by the NTD. In addition, the exact means by which the NTD acts to stimulate cell proliferation and malignant transformation has been obscure since the discovery of c-Myc in the 1980s. Rapid advances in the study of the Mad-Max system revealed that Mad suppressed transcription of target genes through interaction with the corepressor protein mSin3, which recruits histone deacetylases that mediate gene repression (Schreiber-Agus and DePinho, 1998). Given the ability of c-Myc to activate transcription it was widely anticipated that c-Myc would recruit histone acetylases in some manner, but evidence of this was not forthcoming. An apparent recent breakthrough regarding this issue is the discovery of the ATM-related MB2-dependent c-Myc binding protein TRRAP (TRansformation/tRanscription-domain-Associated Protein) (McMahon *et al*., 1998). TRRAP was purified biochemically by using DNA-bound Gal4-Myc fusion proteins as an affinity matrix and a Gal4-MycΔMB2 protein lacking the MB2 region as a negative control. TRRAP is a huge protein of >400 kD that is surprisingly devoid of notable sequence motifs with the exception of an ATM kinase-related domain in its extreme C-terminus. However, this domain lacks enzymatic activity, probably because it lacks key residues which are required for such activity in other ATM family proteins. Thus, this region may serve as a protein-protein interaction domain, perhaps serving as an inhibitor of other ATM family interactions. By expressing sense and antisense fragments of the TRRAP cDNA, evidence was obtained that TRRAP was essential for Ras cotransformation of embryo fibroblasts by both Myc and E1A. Interestingly, TRRAP is in excess to c-Myc and also binds to the transactivation domain of E2F1, suggesting a broader role in cell growth and perhaps transcriptional regulation. A biologically active E2F1 mutant, Y411C, which cannot bind to Rb (which also binds the E2F1 transactivation domain) still interacts with TRRAP in cells, suggesting that TRRAP is an positive cofactor for both Myc and E2F transactivation activity. It will be of particular interest to determine if there is a competitive binding relationship between TRRAP and negative c-Myc NTD regulators or effectors, such as p107, Bin1, or MM-1, or in the case of E2F1, between TRRAP and Rb.

Unlike E2F1 and c-Myc, TRRAP is conserved in evolution to yeast, suggesting a more general role in cell regulation. A recent study identifies the yeast ortholog of TRRAP, Tra1, as a component of the major transcriptional regulatory complex SAGA (Saleh *et al*., 1998). This Tra1-containing complex includes histone acetyltransferases and transcription adaptors/coactivators that in animal cells play an important role in the regulation of enhancers by altering chromatin structure (Grant *et al*., 1998). While the role of Tra1 in SAGA remains to be determined, and connections between TRRAP and mammalian SAGA complexes must be confirmed, it seems eminently likely that TRRAP may provide the long-awaited linkage between c-Myc and a bonified, histone acetylase-containing transcriptional complex. Continued investigations would seem likely to give fundamental insights into how c-Myc controls transcriptional activation, repression, or both, through interactions with TRRAP and probable recruitment of SAGA. At this point, the simplest hypothesis would be that the recruitment of histone acetyltransferase activity to c-Myc/Max binding sites via TRRAP would lead to proximal chromatin remodeling and the recruitment of basal transcription complexes.

Pam

A similar GST fusion technique to generate soluble c-Myc NTD was used for expression screening of a phage library to obtain Pam (Protein Associated with Myc) (Guo *et al*., 1998). Pam is another huge NTD-binding protein with a MW>500 kD. Through *in vitro* and *in vivo* binding assays Pam was shown to associate with c-Myc but not with N-Myc. The region implicated was aa 107–154, spanning the region mentioned above where there is evolutionarily conservation within a single protein but divergence among family members (aa 100–130). Thus, it is tempting to speculate that Pam may have a function that is specific for c-Myc. The functional relevance of Pam in Myc biology has not yet been assessed. However, a notable feature of Pam is the two regions it contains which are similar to RCC1, the nuclear regulator of the cell cycle and chromatin condensation. RCC1 is a guanine nucleotide exchange protein (GEF) for the small nuclear Ras superfamily protein Ran, which regulates traffic through the nuclear pore. It will be interesting to explore possible roles for the Myc/Pam complex in chromatin modeling, given the role of RCC1 in controlling the onset of chromosome condensation (Ohtsubo *et al*., 1989), or in RNA

transport, since Ran mediates this process and c-Myc also regulates gene expression at some nuclear posttranscriptional level other than splicing (Prendergast and Cole, 1989). Analysis of Pam may open up new vistas on Myc regulation or Myc function.

AMY-1

Another novel protein that interacts with the c-Myc NTD is Amy-1 (Associate of c-MYc-1) (Taira *et al.*, 1998). Amy-1 is a small protein of ~11 kD which physically associates with c-Myc in cells via an MB2-containing determinant within aa 48–158. Association of Amy-1 with c-Myc stimulates its E-box-dependent transactivation activity. Amy-1 appears to be subjected to alternate splicing since two types of messages encoding so-called Amy-1S (short) and Amy-1L (long) are expressed, both of which have the same open reading frame and only vary in the amount of 5′-noncoding sequence included. Amy-1 expression is ubiquitous but regulated in a cell cycle-dependent manner, with its highest peak in early S phase. Amy-1 presents another example of a cytosolic Myc-binding protein which translocates into the nucleus at times of increased c-Myc expression and commitment to enter S phase. Interestingly, Amy-1 lacking Myc-binding activity does not translocate into the nucleus appropriately, suggesting that its movement is dependent upon association with Myc. Following S phase, Amy-1 returns to the cytoplasm. Binding experiments suggest that Amy-1 only binds certain phosphorylated forms of c-Myc that are cell cycle controlled. It is hypothesized that Amy-1 is recruited into the nucleus when c-Myc transactivates S phase-specific target genes. As mentioned above, the Myc CTD-binding protein Miz-1 also exclusively exists in the cytoplasm and c-Myc association mediates nuclear import of Miz-1. Therefore, it may be interesting to see if there is any crosstalk between Amy-1 and Miz-1 in terms of cell localization and Myc transcriptional regulation.

Closing perspective

The role of c-Myc in transcriptional regulation is strengthened by the identification of several bonified transcription factors (YY-1, TFII-I, AP-2) and adaptor proteins found in transcriptional regulatory complexes (TRRAP in SAGA) as factors that functionally interact with the CTD and NTD of c-Myc. However, as the number of proteins with which c-Myc interacts begins to enlarge, it appears that the centrality of c-Myc in cell fate regulation reflects dynamic interactions with a wide number of complex regulatory and effector proteins. Analyses of NTD binding proteins argue that the primary level of complexity to be understood is at the NTD network rather than the CTD network, which is responsible for DNA recognition. Thus, the CTD network involves the Mad-Mnt/Rox and Max interactions which oppose Myc function by recruiting mSin3 recruitment and closing chromatin via histone deacetylase action, whereas the NTD network involves a host of proteins, at least one of which is connected directly to a complex containing histone acetylases. Given the ability of several NTD network proteins to modulate transcription, it is tempting to speculate that activation of different classes of target requires global regulation mediated by TRRAP and then specific regulation that is dictated by accessory NTD-binding proteins such as p107, Bin1, MM-1, and Amy-1, which may coordinate targets with cell cycle, apoptosis, etc. In this sense, the novel NTD network proteins may provide valuable tools to sort out the physiological roles of the growing number of cellular genes (currently >30) which have been identified as genetic targets of c-Myc activity (Dang, 1999). While Myc is certainly a *bona fide* transcription factor, its functions appear to extend beyond that of a classical transcription factor; the identification of binding proteins such as Bin1 and Pam suggest unique signaling roles for c-Myc, related to cell fate determination and possibly chromatin modulation or nuclear trafficking actions. Analysis of the NTD network in particular seems likely to breakdown the enigmatic properties of Myc, possibly providing novel insights into basic cell processes as well as offering new avenues for intervention in hyperproliferative diseases where c-Myc is involved, most notably in cancer.

Acknowledgements
This review cites earlier reviews on Myc function to reduce the size of the reference list and we apologize for omitting all the appropriate primary references. We thank members of our laboratory, Kathy Elliott, Kai Ge, and Matt Summers, for critically reading the manuscript. D Sakamuro is supported by a grant from the US Army Breast Cancer Research Program. GC Prendergast is a Pew Scholar in the Biomedical Sciences.

References

Adnane J and Robbins PD. (1995). *Oncogene,* **10,** 381–387.
Alexandrova N, Niklinski J, Bliskovsky V, Otterson GA, Blake M, Kaye FJ and Zajac-Kaye M. (1995). *Mol. Cell. Biol.,* **15,** 5188–5195.
Amati B, Alevizopoulos K and Vlach J. (1998). *Front. Biosci.,* **3,** D250–D258.
Amati B, Brooks MW, Levy N, Littlewood TD, Evan GI and Land H. (1993a). *Cell,* **72,** 233–245.
Amati B and Land H. (1994). *Curr. Opin. Genet. Dev.,* **4,** 102–108.
Amati B, Littlewood TD, Evan GI and Land H. (1993b). *EMBO J.,* **12,** 5083–5087.
Arsura M, Deshpande A, Hann SR and Sonenshein GE. (1995). *Mol. Cell. Biol.,* **15,** 6702–6709.
Askew DS, Ashmun RA, Simmons BC and Cleveland JL. (1991). *Oncogene,* **6,** 1915–1922.
Austen M, Cerni C, Luscher-Firzlaff JM and Luscher B. (1998). *Oncogene,* **17,** 511–520.
Ayer DE and Eisenman RN. (1993). *Genes Dev.,* **7,** 2110–2119.

Ayer DE, Lawrence QA and Eisenman RN. (1995). *Cell*, **80**, 767–776.
Bao J and Zervos AS. (1996). *Oncogene*, **12**, 2171–2176.
Bauer F, Urdaci M, Aigle M and Crouzet M. (1993). *Mol. Cell. Biol.*, **13**, 5070–5084.
Beijersbergen RL, Hijmans EM, Zhu L and Bernards R. (1994). *EMBO J.*, **13**, 4080–4086.
Berberich S, Hyde-deRuyscher N, Espenshade P and Cole M. (1992). *Oncogene*, **7**, 775–779.
Bertwistle D and Ashworth A. (1998). *Curr. Opin. Genet. Dev.*, **8**, 14–20.
Bhatia K, Huppi K, Spangler G, Siwarski D, Iyer R and Magrath I. (1993). *Nat. Genet.*, **5**, 56–61.
Blackwood E, Lüscher B and Eisenman RN. (1992a). *Genes Dev.*, **6**, 71–80.
Blackwood EM, Kretzner L and Eisenman RN. (1992b). *Curr. Opin. Genet. Dev.*, **2**, 227–235.
Bouchard C, Staller P and Eilers M. (1998). *Trends Cell Biol.*, **8**, 202–206.
Bousset K, Henriksson M, Lüscher-Firzlaff JM, Litchfield DW and Lüscher B. (1993). *Oncogene*, **8**, 3211–3220.
Bova GS and Isaacs WB. (1996). *World J. Urol.*, **14**, 338–346.
Brizzio V, Gammie AE and Rose MD. (1998). *J. Cell Biol.*, **141**, 567–584.
Brough DE, Hofmann TJ, Ellwood KB, Townley RA and Cole MD. (1995). *Mol. Cell. Biol.*, **15**, 1536–1544.
Butler MH, David C, Ochoa G-C, Freyberg Z, Daniell L, Grabs D, Cremona O and De Camilli P. (1997). *J. Cell Biol.*, **137**, 1355–1367.
Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB and Jensen RH. (1996). *Cancer Res.*, **56**, 3091–3102.
Chin L, Schreiber-Agus N, Pellicer I, Chen K, Lee HW, Dudast M, Cordon-Cardo C and De Pinho RA. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 8488–8492.
Chou TY, Hart GW and Dang CV. (1995). *J. Biol. Chem.*, **270**, 18961–18965.
Cole MD. (1986). *Ann. Rev. Genet.*, **20**, 361–384.
Crouzet M, Urdaci M, Dulau L and Aigle M. (1991). *Yeast*, **7**, 727–743.
Cultraro CM, Bino T and Segal S. (1997). *Mol. Cell. Biol.*, **17**, 2353–2359.
Dang CV. (1999). *Mol. Cell. Biol.*, **19**, 1–11.
David C, McPherson PS, Mundigl O and de Camilli P. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 331–335.
David C, Solimena M and De Camilli P. (1994). *FEBS Lett.*, **351**, 73–79.
De Pinho RA, Schreiber-Agus N and Alt FW. (1991). *Adv. Cancer Res.*, **57**, 1–46.
Dropcho EJ. (1996). *Ann. Neurol.*, **39**, 659–667.
Eilers M, Picard D, Yamamoto KR and Bishop JM. (1989). *Nature*, **340**, 66–68.
Elliott K, Sakamuro D, Basu A, Du W, Wunner W, Staller P, Gaubatz S, Zhang H, Prochownik E, Eilers M and Prendergast GC. (1999). *Oncogene*, in press.
Evan GI and Littlewood TD. (1993). *Curr. Opin. Genet. Dev.*, **3**, 44–49.
Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell*, **69**, 119–128.
Facchini LM and Penn LZ. (1998). *FASEB J.*, **12**, 633–651.
Ferre-D'Amare A, Prendergast GC, Ziff EB and Burley SK. (1993). *Nature*, **363**, 38–45.
Foley KP, McArthur GA, Queva C, Hurlin PJ, Soriano P and Eisenman RN. (1998). *EMBO J.*, **17**, 774–785.
Frisch SM. (1997). *Bioessays*, **19**, 705–709.
Gallant P, Shiio Y, Cheng PF, Parkhurst SM and Eisenman RN. (1996). *Science*, **274**, 1523–1527.
Gaubatz S, Imhof A, Dosch R, Werner O, Mitchell P, Buettner R and Eilers M. (1995). *EMBO J.*, **14**, 1508–1519.
Gowen LC, Avrutskaya AV, Latour AM, Koller BH and Leadon SA. (1998). *Science*, **281**, 1009–1012.
Grant PA, Sterner DE, Duggan LJ, Workman JL and Berger SL. (1998). *Trends Cell Biol.*, **8**, 193–197.
Grueneberg DA, Henry RW, Brauer A, Novina CD, Cheriyath V, Roy AL and Gilman M. (1997). *Genes Dev.*, **11**, 2482–2493.
Gu W, Bhatia K, Magrath IT, Dang CV and Dalla-Favera R. (1994). *Science*, **264**, 251–254.
Guo Q, Xie J, Dang CV, Liu ET and Bishop JM. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 9172–9177.
Gupta K, Anand G, Yin X, Grove L and Prochownik EV. (1998). *Oncogene*, **16**, 1149–1159.
Gupta S, Seth A and Davis RJ. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 3216–3220.
Haas K, Staller P, Geisen C, Bartek J, Eilers M and Moroy T. (1997). *Oncogene*, **15**, 179–192.
Hakem R, de la Pompa JL, Elia A, Potter J and Mak TW. (1997). *Nat. Genet.*, **16**, 298–302.
Hann SR, Dixit M, Sears RC and Sealy L. (1994). *Genes Dev.*, **8**, 2441–2452.
Hann SR, King MW, Bentley DL, Anderson CW and Eisenman RN. (1988). *Cell*, **52**, 185–195.
Hanson KD, Shichiri M, Follansbee MR and Sedivy JM. (1994). *Mol. Cell. Biol.*, **14**, 5748–5755.
Hassig CA, Fleischer TC, Billin AN, Schreiber SL and Ayer DE. (1997). *Cell*, **89**, 341–347.
Hateboer G, Timmers H, Rustgi AK, Billaud M, Van'tVeer LJ and Bernards R. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 8489–8493.
Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R and Neckers LM. (1987). *Nature*, **328**, 445–448.
Henriksson M, Bakardjiev A, Klein G and Luscher B. (1993). *Oncogene*, **8**, 3199–3209.
Henriksson M and Lüscher B. (1996). *Adv. Cancer Res.*, **68**, 109–182.
Hermeking H and Eick D. (1994). *Science*, **265**, 2091–2093.
Hoang AT, Lutterbach B, Lewis BC, Yano T, Chou T-Y, Barrett JF, Raffeld M, Hann SR and Dang CV. (1995). *Mol. Cell. Biol.*, **15**, 4031–4042.
Holt JT, Redner RL and Nienhuis AW. (1988). *Mol. Cell. Biol.*, **8**, 963–973.
Hopewell R and Ziff EB. (1995). *Mol. Cell. Biol.*, **15**, 3470–3478.
Hurlin PJ, Ayer DE, Grandori C and Eisenman RN. (1994). *Cold Spring Harb. Symp. Quant. Biol.*, **59**, 109–116.
Hurlin PJ, Queva C and Eisenman RN. (1997). *Genes Dev.*, **11**, 44–58.
Iguchi-Ariga SMM, Itani T, Kiji Y and Ariga H. (1987). *EMBO J.*, **6**, 2365–2371.
Kadlec L and Pendergast A-M. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 12390–12395.
Kato GJ and Dang CV. (1992). *FASEB J.*, **6**, 3065–3072.
Kelly K and Siebenlist U. (1986). *Ann. Rev. Immunol.*, **4**, 317–338.
Laherty CD, Yang WM, Sun JM, Davie JR, Seto E and Eisenman RN. (1997). *Cell*, **89**, 349–356.
Land H, Parada LF and Weinberg RA. (1983). *Nature*, **304**, 596–602.
Larsson LG, Bahram F, Burkhardt H and Luscher B. (1997). *Oncogene*, **15**, 737–748.
Lemaitre JM, Buckle RS and Mechali M. (1996). *Adv. Cancer Res.*, **70**, 95–144.
Li L, Nerlov C, Prendergast G, MacGregor D and Ziff EB. (1994). *EMBO J.*, **13**, 4070–4079.
Lutterbach B and Hann SR. (1994). *Mol. Cell. Biol.*, **14**, 5510–5522.
Mäkelä TP, Koskinen PJ, Västrik I and Alitalo K. (1992). *Science*, **256**, 373–377.

Mao NC, Steingrimsson E J D, Ruiz J, Wasserman W, Copeland NG, Jenkins NA and Prendergast GC. (1999). *Genomics*, in press.
Martel C, Lallemand D and Cremisi C. (1995). *Oncogene*, **10**, 2195–2205.
McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD and Cole MD. (1998). *Cell*, **94**, 363–374.
Meichle A, Philipp A and Eilers M. (1992). *Biochim. Biophys. Acta*, **1114**, 129–146.
Meroni G, Reymond A, Alcalay M, Borsani G, Tanigami A, Tonlorenzi R, Nigro CL, Messali S, Zollo M, Ledbetter DH, Brent R, Ballabio A and Carrozzo R. (1997). *EMBO J.*, **16**, 2892–2906.
Mori K, Maeda Y, Kitaura H, Taira T, Iguchi-Ariga SMM and Ariga H. (1998). *J. Biol. Chem.*, **273**, 29794–29800.
Morriss-Kay GM. (1996). *Bioessays*, **18**, 785–788.
Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R and Fassler R. (1997). *Genes Dev.*, **11**, 1938–1948.
Mukherjee B, Morgenbesser SD and De PR. (1992). *Genes Dev*, **6**, 1480–1492.
Munn AL, Stevenson BJ, Geli MI and Riezman H. (1995). *Mol. Biol. Cell*, **6**, 1721–1742.
Negorev D, Reithman H, Wechsler-Reya R, Sakamuro D, Prendergast GC and Simon D. (1996). *Genomics*, **33**, 329–331.
Ohtsubo M, Okazaki H and Nishimoto T. (1989). *J. Cell. Biol.*, **109**, 1389–1397.
Owen DJ, Wigge P, Vallis Y, Moore JD, Evans PR and McMahon HT. (1998). *EMBO J.*, **17**, 5273–5285.
Packham G and Cleveland JL. (1994). *Mol. Cell. Biol.*, **14**, 5741–5747.
Papas TS and Lautenberger JA. (1985). *Nature*, **318**, 237.
Peukert K, Staller P, Schneider A, Carmichael G, Hanel F and Eilers M. (1997). *EMBO J.*, **16**, 5672–5686.
Philipp A, Schneider A, Väsrik I, Finke K, Xiong Y, Beach D, Alitalo K and Eilers M. (1994). *Mol. Cell. Biol.*, **14**, 4032–4043.
Prendergast GC. (1997). In: Yaniv, M. and Ghysdael, J. (eds). *Oncogenes as Transcriptional Regulators*. Birkhauser Verlag: Boston, pp 1–28.
Prendergast GC and Cole MD. (1989). *Mol. Cell. Biol.*, **9**, 124–134.
Prendergast GC, Hopewell R, Gorham B and Ziff EB. (1992). *Genes Dev.*, **6**, 2429–2439..
Prendergast GC, Lawe D and Ziff EB. (1991). *Cell*, **65**, 395–407.
Prendergast GC and Ziff EB. (1991). *Science*, **251**, 186–189.
Prendergast GC and Ziff EB. (1992). *Trends Genet.*, **8**, 91–96.
Prochownik EV and Van Antwerp ME. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 960–964.
Ralston R. (1991). *Nature*, **353**, 866–869.
Ramjaun AR and McPherson PS. (1998). *J. Neurochem.*, **70**, 2369–2376.
Ramjaun AR, Micheva KD, Bouchelet I and McPherson PS. (1997). *J. Biol. Chem.*, **272**, 16700–16706.
Riggs KJ, Saleque S, Wong KK, Merrell KT, Lee JS, Shi Y and Calame K. (1993). *Mol. Cell. Biol.*, **13**, 7487–7495.
Roy A, Carruthers C, Gutjahr T and Roeder RG. (1993a). *Nature*, **365**, 359–361.
Roy AL, Du H, Gregor PD, Novina CD, Martinez E and Roeder RG. (1997). *EMBO J.*, **16**, 7091–7104.
Roy AL, Malik S, Meisterernst M and Roeder RG. (1993b). *Nature*, **365**, 355–361.
Ruley HE. (1983). *Nature*, **304**, 602–606.
Rustgi AK, Dyson N and Bernards R. (1991). *Nature*, **352**, 541–544.
Ryan KM and Birnie GD. (1997a). *Biochem. J.*, **325**, 79–85.
Ryan KM and Birnie GD. (1997b). *Oncogene*, 2835–2843.
Sakamuro D, Elliott K, Wechsler-Reya R and Prendergast GC. (1996). *Nature Genet.*, **14**, 69–77.
Sakamuro D, Eviner V, Elliott K, Showe L, White E and Prendergast GC. (1995). *Oncogene*, **11**, 2411–2418.
Saleh A, Schieltz D, Ting N, McMahon SB, Litchfield DW, Yates JRR, Lees-Miller SP, Cole MD and Brandl CJ. (1998). *J. Biol. Chem.*, **273**, 26559–26565.
Salghetti SE, Kim SY and Tansey WP. (1999). *EMBO J.*, **18**, 717–726.
Sawyers CL, Callahan W and Witte ON. (1992). *Cell*, **70**, 901–910.
Schreiber-Agus N, Chin L, Chen K, Torres R, Rao G, Guida P, Skoultchi AI and De Pinho RA. (1995). *Cell*, **80**, 777–786.
Schreiber-Agus N and DePinho RA. (1998). *Bioessays*, **20**, 808–818.
Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J and Livingston DM. (1997a). *Cell*, **90**, 425–435.
Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T and Livingston DM. (1997b). *Cell*, **88**, 265–275.
Seth A, Gupta S and Davis RJ. (1993). *Mol. Cell. Biol.*, **13**, 4125–4136.
Shibuya H, Yoneyama M, Ninomiya-Tsuji J, Matsumoto K and Taniguchi T. (1992). *Cell*, **70**, 57–67.
Showe LC, Ballantine M, Nishikura K, Erikson J, Kaji H and Croce CM. (1985). *Mol. Cell. Biol.*, **5**, 501–509.
Shrivastava A, Saleque S, Kalpana GV, Artandi S, Goff SP and Calame K. (1993). *Science*, **262**, 1889–1892.
Shrivastava A, Yu J, Artandi S and Calame K. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 10638–10641.
Sklar MD, Thompson E, Welsh MJ, Liebert M, Harney J, Grossman HB, Smith M and Prochownik EV. (1991). *Mol. Cell. Biol.*, **11**, 3699–3710.
Smith-Sorensen B, Hijmans EM, Beijersbergen RL and Bernards R. (1996). *J. Biol. Chem.*, **271**, 5513–5518.
Sommer A, Bousset K, Kremmer E, Austen M and Luscher B. (1998). *J. Biol. Chem.*, **273**, 6632–6642.
Spencer CA and Groudine M. (1991). *Adv. Cancer Res.*, **56**, 1–48.
Steiner P, Rudolph B, Muller D and Eilers M. (1996). *Prog. Cell Cycle Res.*, **2**, 73–82.
Sterner JM, Tao Y, Kennett SB, Kim HG and Horowitz JM. (1996). *Cell Growth Diff.*, **7**, 53–64.
Stone J, de Lange T, Ramsay G, Jakobovits E, Bishop JM, Varmus H and Lee W. (1987). *Mol. Cell. Biol.*, **7**, 1697–1709.
Taira T, Maeda J, Onishi T, Kitaura H, Yoshida S, Kato H, Ikeda M, Tamai K, Iguchi-Ariga SM and Ariga H. (1998). *Genes Cells*, **3**, 549–565.
Tsutsui K, Maeda Y, Tsutsui K, Seki S and Tokunaga A. (1997). *Biochem. Biophys. Res. Comm.*, **236**, 178–183.
Vastrik I, Makela TP, Koskinen PJ and Alitalo K. (1995). *Oncogene*, **11**, 553–560.
Vriz S, Lemaitre J-M, Leibovici M, Thierry N and Méchali M. (1992). *Mol. Cell. Biol.*, **12**, 3548–3555.
Wagner AJ, Kokonitis JM and Hay N. (1994). *Genes Dev.*, **8**, 2817–2830.
Wagner AJ, LeBeau MM, Diaz MO and Hay N. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 3111–3115.
Wang Q, Zhang H, Kajino K and Greene MI. (1998). *Oncogene*, **17**, 1939–1948.
Wechsler-Reya R, Elliott K and Prendergast GC. (1998). *Mol. Cell. Biol.*, **18**, 566–575.
Wechsler-Reya R, Sakamuro D, Zhang J, Duhadaway J and Prendergast GC. (1997). *J. Biol. Chem.*, **272**, 31453–31458.
Wigge P, Vallis Y and McMahon HT. (1997). *Curr. Biol.*, **7**, 554–560.

Yano T, Sander CA, Clark HM, Dolezal MV, Jaffe ES and Raffeld M. (1993). *Oncogene*, **8**, 2741–2748.

Yin X, Grove L and Prochownik EV. (1998). *Oncogene*, **16**, 2629–2637.

Yuan J, Tirabassi RS, Bush AB and Cole MD. (1998). *Oncogene*, **17**, 1109–1118.

Zhu MH, John S, Berg M and Leonard WJ. (1999). *Cell*, **96**, 121–130.

Oncogene (1999) 18, 2967–2987

http://www.stockton-press.co.uk/onc

Mechanisms of apoptosis by c-Myc

George C Prendergast*,[1]

[1]*The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania, PA 19104, USA*

Much recent research on c-Myc has focused on how it drives apoptosis. c-Myc is widely known as a crucial regulator of cell proliferation in normal and neoplastic cells, but until relatively recently its apoptotic properties, which appear to be intrinsic, were not fully appreciated. Its death-dealing aspects have gained wide attention in part because of their potential therapeutic utility in advanced malignancy, where c-Myc is frequently deregulated and where novel modalities are badly needed. Although its exact function remains obscure, c-Myc is a transcription factor and advances have been made in characterizing target genes which may mediate its apoptotic properties. Candidate regulators and effectors are also emerging. Among recent findings are connections to the CD95/Fas and TNF pathways and roles for the tumor suppressor p19ARF and the c-Myc-interacting adaptor protein Bin1 in mediating cell death. In this review I summarize the data establishing a role for c-Myc in apoptosis in diverse settings and present a modified dual signal model for c-Myc function. It is proposed that c-Myc induces apoptosis through separate 'death priming' and 'death triggering' mechanisms in which 'death priming' and mitogenic signals are coordinated. Investigation of the mechanisms that underlie the triggering steps may offer new therapeutic opportunities.

Keywords: cell death; transformation; cell cycle; transcription; signal transduction

Introduction

c-Myc is a member of the Myc family of b/HLH/LZ proteins which regulate cell proliferation and apoptosis. Expression of Myc proteins is deregulated in approximately one-third of human cancers through a variety of mechanisms (Cole, 1986; Kelly and Siebenlist, 1986; Spencer and Groudine, 1991). Overexpression of Myc is especially common in certain advanced cancers, such as hormone-independent adenocarcinomas of the breast and prostate, where it is associated with poor prognosis (Berns *et al.*, 1992; Borg *et al.*, 1992; Hehir *et al.*, 1993; Kreipe *et al.*, 1993; Shiu *et al.*, 1993; Watson *et al.*, 1993; Strohmeyer and Slamon, 1994; Bova and Isaacs, 1996; Cher *et al.*, 1996; Jenkins *et al.*, 1997). However, since deregulation is sufficient for oncogenic activation, the involvement of Myc in late stage cancers may be even broader than suggested by these studies.

*Correspondence: GC Prendergast

c-Myc has a central and necessary role in the proliferation of normal cells. Following mitogenic stimulation of quiescent cells, Myc is rapidly induced and remains elevated, suggesting that it is required for continuous cell growth. Although its exact function remains unclear, Myc appears to be needed at several points during the cell cycle (Evan and Littlewood, 1993). Induction of Myc is sufficient to drive quiescent cells into the cell cycle (Eilers *et al.*, 1989), while inhibition of Myc can block mitogenic signals and facilitate cell differentiation (Heikkila *et al.*, 1987; Holt *et al.*, 1988; Sklar *et al.*, 1991; Sawyers *et al.*, 1992; Hanson *et al.*, 1994). Recent reviews have comprehensively surveyed the numerous investigations aimed at understanding how c-Myc drives cell cycle progression, proliferation, and malignant transformation (Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Ryan and Birnie, 1996; Grandori and Eisenman, 1997; Amati *et al.*, 1998; Bouchard *et al.*, 1998; Facchini and Penn, 1998; Dang, 1999; and reviews in this issue).

In the early 1990s Cleveland and Evan and their colleagues established definitively that c-Myc can also activate apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992), a cell suicide program that is intrinsic to metazoan cells (Kerr *et al.*, 1972; Wyllie, 1993). Toxic effects of elevated c-Myc expression were noted anecdotally by many investigators in the 1980s and reported by several laboratories (e.g. see studies cited in Packham and Cleveland, 1995). However, before apoptosis became accepted as a *bona fide* cellular process, the toxicity of deregulated c-Myc was not conceptualized as a potential function. An early study by Wyllie and colleagues was a harbinger of this paradigm shift (Wyllie *et al.*, 1987). The capacity of c-Myc to drive apoptosis *in vitro* was first credibly established under growth limiting conditions where its expression was enforced and uncoupled from growth factor controls. Thus, following growth factor withdrawal, cells that contain normal c-Myc downregulate its expression and exit the cell cycle, whereas cells where c-Myc is enforced maintain its expression and undergo apoptosis (Evan *et al.*, 1992). A complete survey of initial investigations of the apoptotic properties of c-Myc is provided in three initial excellent reviews which helped to frame the questions, models, terminology, and discussion in this area (Harrington *et al.*, 1994b; Evan *et al.*, 1995; Packham and Cleveland, 1995). Among the more important initial questions were whether the death-dealing aspect of c-Myc was due to an intrinsic function or not and how this aspect was regulated and mediated. Here we consider advances made on these issues, beginning by introducing c-Myc, the basic machinery of apoptosis, and the significance of apoptosis to cancer. The findings of biological studies are outlined and a modified version of the dual signal model for c-Myc function is framed. Progress in

identifying regulatory and effector mechanisms involved in apoptosis by c-Myc are surveyed and some pressing questions are posed.

c-Myc structure and function

While its exact role in the cell remains a subject of some debate, an extensive body of work argues strongly that c-Myc acts chiefly in the guise of a transcription factor (Henriksson and Lüscher, 1996; Lemaitre *et al.*, 1996; Prendergast, 1997; Facchini and Penn, 1998). c-Myc is the main member of a nonredundant family of Myc proteins that also includes N-Myc, L-Myc, S-Myc, and B-Myc. *myc* genes appear to have arisen relatively late in evolution and functional homologs have not been definitively identified in organisms more primitive than *Drosophila* (Gallant *et al.*, 1996; Schreiber-Agus *et al.*, 1997). Like other Myc proteins, c-Myc is roughly tripartite in organization, with the crucial regions needed for proliferation, apoptosis, and transcriptional activities located in its terminal domains (see Figure 1). The C-terminal domain (CTD) includes a basic domain/helix–loop–helix/leucine zipper (b/HLH/Z) motif that mediates oligomerization through the HLH/Z region and specific DNA recognition of CACGTG E box motifs present in all target genes through the basic domain (Blackwell *et al.*, 1990; Halazonetis and Kandil, 1991; Kerkhoff *et al.*, 1991; Prendergast and Ziff, 1991). Overexpression of heterologous dimers of the c-Myc basic region dominantly interfered with malignant transformation by c-Myc, offering initial genetic proof that CACGTG is a physiological Myc recognition site (Prendergast and Ziff, 1991). However, Myc proteins do not homo-oligomerize in cells. Physiological DNA binding and biological activity depends upon hetero-oligomerization with Max, a small, nonredundant, and ubiquitously expressed protein comprised essentially of a b/HLH/Z domain (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Wenzel *et al.*, 1991; Berberich *et al.*, 1992; Blackwood *et al.*, 1992; Kato *et al.*, 1992; Reddy *et al.*, 1992; Ferre-D'Amare *et al.*, 1993; Ma *et al.*, 1993). Phosphorylation and alternate splicing proximal to the basic region inhibit the ability of Max homodimers to associate with DNA in cells (Berberich and Cole, 1992; Prochownik and Van Antwerp, 1993; Zhang *et al.*, 1997). Max association and DNA binding are required for transcriptional activation of target genes by c-Myc as well as its ability to drive proliferation, malignant cell transformation, and apoptosis (Amati *et al.*, 1992; Kretzner *et al.*, 1992a,b; Mäkelä *et al.*, 1992; Mukherjee *et al.*, 1992; Prendergast *et al.*, 1992; Amati *et al.*, 1993a,b; Gu *et al.*, 1993). Access to CACGTG binding sites in target genes may be regulated by CpG methylation of the central dinucleotide (Prendergast and Ziff, 1991; Prendergast *et al.*, 1991). The CTD interacts with proteins in addition to Max including YY-1, AP-2, BRCA-1, TFII-I, and Miz-1, which all appear to influence DNA binding by c-Myc/Max complexes in some fashion (see Lüscher and Larsson, this issue). AP-2 has been implicated in suppressing apoptosis by c-Myc (Moser *et al.*, 1997) but possible roles for the other factors in cell death has not been examined. In summary, the CTD is devoted to specific DNA recognition by c-Myc and regulation of its access to specific DNA binding sites.

The N-terminal domain (NTD) includes the transcriptional activation domain (TAD) of Myc and probably other functions. Two ~20 aa segments in the NTD termed Myc boxes 1 and 2 (MB1 and MB2) are conserved in all Myc family proteins and are crucial for all biological activities. The first glimpse of the function of this region was provided by the observation that the NTD activated transcription when fused to a heterologous DNA binding domain (Kato *et al.*, 1990). This initial finding was later validated by demonstrations that the c-Myc/Max complex could activate transcription from artificial promoters as well as from candidate target genes (Amati *et al.*, 1992; Kretzner *et al.*, 1992a; Bello-Fernandez *et al.*, 1993; Benvenisty *et al.*, 1993; Crouch *et al.*, 1993; Jansen-Durr *et al.*, 1993; Reisman *et al.*, 1993; Wagner *et al.*, 1993a; Gaubatz *et al.*, 1994). The mechanism through which the NTD activates transcription in unclear. A good candidate, however, has emerged in the ATM-related protein TRRAP, an NTD-binding protein that is part of the SAGA complex which includes histone deacetylases implicated in activation events (Grant *et al.*, 1998; McMahon *et al.*, 1998; Saleh *et al.*, 1998). The activation activity of the NTD may also be influenced by interactions with the retinoblastoma (Rb)-related protein p107 (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994; Hoang *et al.*, 1995), whose binding to c-Myc may be modulated by cyclin D/CDK4 phosphorylation (Hass *et al.*, 1997), or with the adaptor protein and tumor suppressor Bin1 (Sakamuro *et al.*, 1996; Elliott *et al.*, 1999). The NTD is also reported to interact with α-tubulin (Alexandrova *et al.*, 1995), PAM, a large protein which includes RCC1-like repeats suggesting a role in chromatin regulation (Guo *et al.*, 1998), MM-1, a nucleocytoplasmic adaptor protein that inhibits transactivation by c-Myc, and AMY-1, a small protein reported to potentiate the transactivation activity of c-Myc (Taira *et al.*, 1998). In addition to harboring a transactivation domain, the NTD also mediates transcriptional repression (Kaddurah-Daouk *et al.*, 1987; Suen and Hung, 1991; Yang *et al.*, 1991, 1993; Jansen-Durr *et al.*, 1993; Roy *et al.*, 1993; Li *et al.*, 1994; Philipp *et al.*, 1994; Lee *et al.*, 1996; Tikhonenko *et al.*, 1997). Repression of growth arrest genes may be one way in which Myc promotes growth and perhaps apoptosis (Lee *et al.*, 1996). Myc box 2 appears to be crucial for repression and transformation (Li *et al.*, 1994; Brough *et al.*, 1995; Lee *et al.*, 1996; Xiao *et al.*, 1998). Activation is thought to be important for all biological activities, but two recent studies using Rat1 fibroblasts nullizygous for c-*myc*, which are viable and replicatively competent but which proliferate slowly (Mateyak *et al.*, 1997), have challenged the role of the transactivation properties of c-Myc in cell proliferation (Bush *et al.*, 1998; Xiao *et al.*, 1998). Roles for a subset of c-Myc target genes implicated in apoptosis are discussed below. For a full listing and consideration of the >30 reported target genes and their potential roles in mediating the various biological effects of c-Myc the reader is directed to a recent comprehensive review on this topic (Dang, 1999).

Apoptosis: the basic 'executioner' machinery and key pathways to it in cancer

Apoptosis (meaning 'a dropping off') is an intrinsic cell suicide program that is universal in metazoan organisms. Strictly speaking, apoptosis is defined by morphological features. It is characterized by cell shrinkage, violent blebbing of the plasma membrane and chromatin condensation (Wyllie, 1993). Unlike a necrotic cell, the apoptotic cell does not leak internal contents and it does not induce an inflammatory response. The pieces of the cell which remain when the program is complete are termed apoptotic bodies. Many features of apoptosis are not so readily detected *in vivo* as might be expected because soon after the death program is engaged cells are quickly recognized by macrophages and engulfed. Neighboring cells may also perform this act. Engulfment is triggered by the appearance of phosphatidylserine on the outer leaflet of the plasma membrane of the dying cell, a process which is catalyzed by an activated 'flipase'. Apoptosis is a

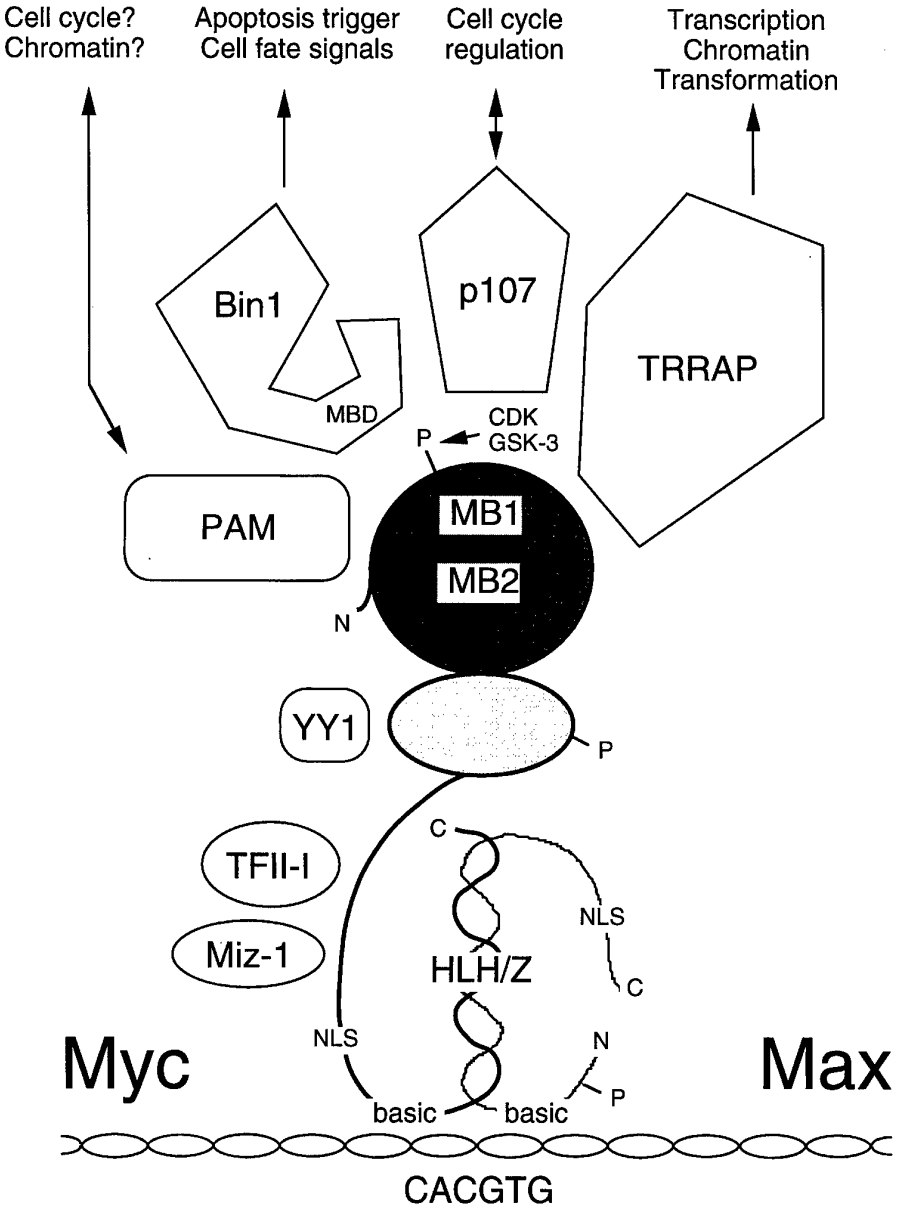


Figure 1 Myc structure and major binding proteins. The CTD harbors the b/HLH/Z motif for oligomerization and DNA binding and the NTD harbors evolutionarily conserved 'Myc boxes' 1 and 2 (MB1 and MB2). Both terminal domains are required to regulate transcription of target genes, drive cell cycle transit, cause malignant transformation, and activate apoptosis. Max is a physiological partner for c-Myc that binds to the CTD and mediates DNA binding as a heterodimer with c-Myc. Max is necessary for both transformation and apoptosis. Miz-1, TFII-I, and other proteins also associate with the CTD. MB1 is the target of phosphorylation by Cdk and Gsk-3. The central domain between the NTD and CTD is largely dispensable. The adaptor protein Bin1 and the ATM-related protein TRRAP are NTD-binding proteins implicated in apoptosis and transformation, respectively. TRRAP may mediate transcriptional effects since it is part of the SAGA complex, which contains histone acetylases that globally control chromatin state and transcription. p107 may co-ordinate c-Myc activity with the cell cycle. PAM has RCC1-like repeats suggesting a role in chromatin modeling or cell cycle

default which is engaged unless cells receive sufficient survival signals to negate it (Raff, 1992).

Key advances in the molecular understanding of apoptosis were realized by genetic analysis of the development of the soil nematode *Caenorhabditis elegans* (Ellis *et al.*, 1991). The lineage and fate of all the somatic cells in this genetically malleable organism are known and 131 cells die by apoptosis during development. By analysing mutant nematodes that had abnormal numbers of cells Horvitz and colleagues were able to clone genes that controlled cell death (*ced*, cell death abnormal). Three genes cloned in this way, *ced-3*, *ced-4*, and *ced-9*, proved to encode the central regulatory machinery of apoptosis, sometimes referred to as the 'executioner' machinery, which is conserved in evolution between *C. elegans* and mammals (Yuan, 1996). Genetic analysis demonstrated that *ced-9* suppresses activation of *ced-3* by *ced-4* through direct biochemical interactions (Metzstein *et al.*, 1998). The blueprint provided by *C. elegans* has helped guide mammalian cell studies. Figure 2 presents the components of the fundamental executioner machinery and selected key pathways which regulate it.

ced-9 proved to be homologous to the mammalian protein Bcl-2, an antiapoptotic protein cloned from a follicular B cell tumor which locates mainly to the outer membrane of the mitochondria (Adams and Cory, 1998). In mammalian cells, the Bcl-2 family includes antiapoptotic members such as Bcl-X_L and proapoptotic members such as Bax, Bak, Bid, and Bad (Adams and Cory, 1998). Current understanding of this important class of cell survival regulators suggest that they control the status of the permeability transition (PT) in mitochondria and the efflux of ions or proteins, most notably the electron transport protein cytochrome c (Chao and Korsmeyer, 1998; Green and Reed, 1998). Possibly through their ability to form multimeric pore-like complexes, antiapoptotic members including Bcl-X_L and Bcl-2 are believed to inhibit efflux whereas a subset of proapoptotic members including Bax are believed to oppose this process and promote efflux (Adams and Cory, 1998). Most proapoptotic members, such as Bad and Bid, lack features suggesting pore-forming roles and instead have a single ligand binding domain (BH3 domain) which may act by displacing proapoptotic proteins from antiapoptotic partners and promoting the pore-forming action of the former (Kelekar and Thompson, 1998). Bcl-2 family proteins interact with each other and the ratio of active proapoptotic to antiapoptotic proteins is a critical determinant of cell survival. There appear to be preferences for association, for example, Bcl-2/Bax and Bcl-X_L/Bad. The function of some Bcl-2 family proteins is regulated by posttranslational phosphorylation (Chao and Korsmeyer, 1998). For example, microtubule disrupters cause Bcl-2 to become phosphorylated in tumor cells by Raf (Blagosklonny *et al.*, 1997), whereas Bad is

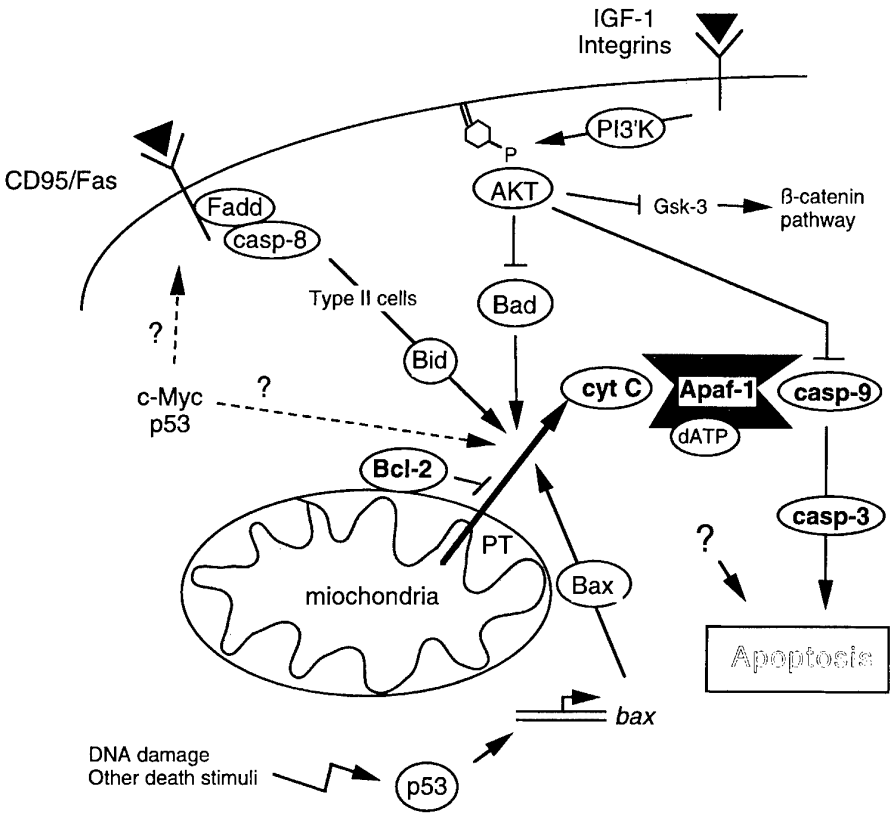


Figure 2 Basic apoptotic machinery and some regulation relevant to cancer. Key components of the basic machinery in mammalian cells are noted in bold type. Three regulatory pathways germane to c-Myc and cancer are indicated, including the p53-Bax, AKT-Bad and AKT-caspase-9, and Type II pathways used by the death receptor CD95 (see text). Proapoptotic signals produced by c-Myc and p53 may act at several levels (see text). Apoptosis by c-Myc may involve separate caspase-3-dependent and caspase-independent signals

downregulated by activation of the phosphatidylinositol-3′-kinase (PI3′K)-Akt kinase cell survival pathway (Datta *et al.*, 1997).

ced-3 was found to be similar to IL-1β-converting enzyme (ICE), a proenzyme processing cysteine protease that proved to have proapoptotic properties (Thornberry and Lazebnik, 1998). The ICE-related proteases, of which there are currently 14, are characterized by cysteine specificity and have been termed caspases (Thornberry and Lazebnik, 1998). Caspases are synthesized as inactive precursors and activated either by autocleavage, when concentrated at certain loci in cells, or by cleavage by another caspase, which in either case generates an active heterodimer composed of $\sim$10 kD and $\sim$20 kD subunits. Caspases have long or short N-terminal prodomains. Those with long prodomains (regulatory caspases) cleave and activate those with short prodomains (effector caspases). Caspases of special relevance to cancer are caspase-3, which is responsible for chromatin collapse and DNA degradation (Woo *et al.*, 1998), caspase-8, which is the first caspase in the CD95/Fas pathway (Ashkenazi and Dixit, 1998), and caspase-9, which is activated by Bcl-2 family-dependent events at mitochondria and is responsible for cleavage and activation of caspase-3 (Li *et al.*, 1997; Zou *et al.*, 1997). Notably, oncogenes including c-Myc potentiate the activation of caspase-9 in cells through as yet undefined mechanisms (Fearnhead *et al.*, 1997, 1998). More than 40 caspase substrates have been defined whose cleavage is believed to be important to cell demise. Among the most crucial substrates is the apoptotic DNA endonuclease DFF/CAD, which is expressed as an inactive precursor complex with the inhibitor ICAD and which is cleaved and activated by caspase-3 (Liu *et al.*, 1997; Enari *et al.*, 1998). A wide variety of caspase inhibitors have been characterized, increasing the complexity of caspase regulation in mammalian cells (Thornberry and Lazebnik, 1998).

In an elegant set of biochemical experiments Wang and colleagues identified the mammalian homolog of *ced-4*, termed Apaf-1, through its ability to activate caspase-3 (Zou *et al.*, 1997). Necessary cofactors that purified in a complex with Apaf-1 were cytochrome c, a key member of the electron transport chain located in mitochondria, dATP, and caspase-9 (Li *et al.*, 1997). Apaf-1 is cytosolic and cytochrome c is normally found in mitochondria, implying that mitochondria-dependent cytochrome c release is necessary for Apaf-1 to promote apoptosis. The link to mitochondria provides the connection to Bcl-2 family proteins which are believed to act there as ion and/or protein channels or possibly as regulators of such channels (Green, 1998; Green and Reed, 1998; Reed *et al.*, 1998). As mentioned above, cytochrome c release can be elicited by proapoptotic members of the Bcl-2 family and also by calcium (Eskes *et al.*, 1998). In addition, biochemical analyses have implicated adenovirus E1A, c-Myc, and p53 in promoting cytochrome c release and caspase activation through some undefined mechanism(s) (Fearnhead *et al.*, 1997, 1998; Ding *et al.*, 1998). The relevant connections and whether they involve calcium or Bcl-2 family proteins should emerge shortly. However, in whatever way it is released, cytochrome c association with Apaf-1 in the presence of bound dATP leads to a recruitment and aggregation of caspase-9 that triggers its autocleavage and activation. Only mitochondrially assembled (i.e. heme-bound) cytochrome c will stimulate Apaf-1, reinforcing the role of mitochondria in regulating activation of caspase-9. The biochemical complex carrying out this process (minimally comprised of Apaf-1, cytochrome c, dATP, and caspase-9) has been termed an 'apoptosome' since it is sufficient to elicit apoptosis in cells. Mammalian Apaf-1 includes in its C-terminal domain a set of WD40 repeats which may regulate the ability to activate caspase-9 (Hu *et al.*, 1998). Consistent with the *C. elegans* blueprint, Bcl-X_L can form a ternary complex with Apaf-1 and caspase-9 (Pan *et al.*, 1998). Additional Bcl-2 family proteins have been identified that interact directly with Apaf-1, termed Diva and Boo. Diva is a proapoptotic protein that acts via a BH3-independent mechanism (Inohara *et al.*, 1998). Boo is an antiapoptotic protein whose interaction with Apaf-1 is displaced by the BH3-dependent proapoptotic proteins Bak and Bik. A central role of Apaf-1 in mammalian cell apoptosis and mouse development has been demonstrated and, as expected, cells lacking Apaf-1 exhibit defects in the ability to activate apoptosis by many stimuli (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998). However, some Apaf-1-independent apoptosis induced by certain stimuli (e.g. staurosporine) raised the possibility that additional Apaf-1-like proteins may exist.

Three pathways which lead to the basic executioner machinery deserve special mention with regard to c-Myc and cancer (see below). The first is the p53-Bax pathway. p53 is a checkpoint regulator and tumor suppressor that is activated by DNA damage and certain other stresses (Levine, 1993). Under catastrophic circumstances p53 will trigger apoptosis in part by stimulating expression of Bax, which is a target of transcriptional activation by p53 (Miyashita *et al.*, 1994; Selvakumaran *et al.*, 1994a; Miyashita and Reed, 1995). Elevation of Bax by p53 is not a sufficient cause for the latter to drive apoptosis; this also requires an undefined signal from the specific apoptosis effector domain of p53 (Sakamuro *et al.*, 1997). Nevertheless, Bax elevation tilts mitochondria toward cytochrome c release and activation of caspase-9, which stimulates caspase-3 activity and cell demise. A second pathway linked to the executioner machinery in cancer cells is the PI3′K-AKT survival pathway. This pathway can be activated by a variety of stimuli, including integrin-dependent cell adhesion, ligation of the receptors for insulin-like growth factor-1 (IGF1) or IL-3, and activated Ras (Datta *et al.*, 1997; del Peso *et al.*, 1997; Franke *et al.*, 1997; Frisch and Ruoslahti, 1997; Khwaja *et al.*, 1997; King *et al.*, 1997; Liu *et al.*, 1998). Two substrates in the executioner machinery for the AKT kinase which have been identified are Bad and caspase-9 (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998). A recent report suggests that a BAD may also be targeted for phosphorylation via a PI3′K-independent pathway involving MEK (Scheid and Duronio, 1998). AKT phosphorylation of Bad leads to loss of its proapoptotic activity, probably by affecting its ability to associate with Bcl-X_L. Similarly, AKT phosphorylation of caspase-9 reduces its proteolytic activity (Cardone *et al.*, 1998), illustrating two levels at which

AKT targets the executioner machinery for inhibition. A third set of related executioner pathways of interest to c-Myc are those activated by cell surface ligation of the death receptors CD95/Fas, which depending on cell type either bypasses or involves mitochondria (Type I or Type II; Scaffidi *et al.*, 1998; Green, 1998), or certain members of the TNF-R family, which depending on receptor type may stimulate both death and NF-KB-dependent survival signals (Ashkenazi and Dixit, 1998). Death in each case is mediated by direct activation of caspase-8, which autocleaves following its recruitment and aggregation by the receptor-binding death effector domain (DED) adaptor proteins FADD to CD95/Fas or FADD+TRADD to TNF-R. Depending on cell and receptor type, robust activation of caspase-8 occurs, leading directly to caspase-3 activation and cellular demise, or weaker activation of caspase-8 occurs, followed by cleavage of the proapoptotic Bcl-2 protein Bid, which stimulates cytochrome c release and activation of caspase-3 via caspase-9 cleavage (Li *et al.*, 1998; Luo *et al.*, 1998). CD95/Fas and TNF-R pathways may be mechanistically crucial in many cell death settings, including cancer.

Apoptosis: its significance in cancer

Apoptosis has many roles in development and homeostasis and its dysregulation is a hallmark of many diseases (Peter *et al.*, 1997). One of its crucial roles is to limit inappropriate cell proliferation that can lead to cancer (Evan and Littlewood, 1998). Apoptosis can stanch such proliferation but apoptotic mechanisms are progressively eliminated during neoplastic progression (Williams, 1991). Substantial clinical evidence argues that this progressive elimination is a crucial step in malignant conversion, for example, in the progression of prostate carcinoma to hormone independence (Kyprianou *et al.*, 1990; McDonnell *et al.*, 1992; Brändström *et al.*, 1994; Raffo *et al.*, 1995). This is not due to loss or inactivation of the basic machinery of apoptosis, which remains intact even in advanced malignancies (Martin and Green, 1995). For example, *in vitro* studies of estrogen-independent breast tumor cells have indicated that they retain an intrinsic capacity for apoptosis, even though they are resistant to hormone deprival (Kyprianou *et al.*, 1991; Armstrong *et al.*, 1992). Neoplastic cells are resistant to apoptosis apparently because they lack or suppress the regulatory mechanisms required to activate the basic executioner machinery. Mutation of p53 or elevation of antiapoptotic members of the Bcl-2 family are paradigms in cancer. As outlined above, activation of AKT by various means in cancer cells is also likely to be important (Franke *et al.*, 1997; Frisch and Ruoslahti, 1997; Ruggeri *et al.*, 1998; Wu *et al.*, 1998). However, the executioner machinery remains intact in cells transformed by c-Myc, which may offer an Achilles' heel to exploit. Consistent with this notion, latent but as yet undefined activators of caspases are clearly present in cells c-Myc-transformed cells but not in normal cells (Fearnhead *et al.*, 1997; Ding *et al.*, 1998). Thus, the interface between c-Myc and the executioner machinery offers a logical realm to identify novel therapeutic strategies.

c-Myc induces apoptosis

c-Myc is sufficient and necessary for apoptosis under certain conditions. The initial observations establishing this were made in IL-3-dependent murine myeloid 32D cells, primary and established rat fibroblasts, and T cell hybridomas. Askew *et al.* (1991) showed that constitutive expression of c-Myc markedly accelerated apoptosis of 32D cells denied IL3. Death was not restricted to a particular phase of the cell cycle but occurred in all phases, and c-Myc continued to drive S phase entry of cells which did not immediately commit suicide. Evan *et al.* (1992) demonstrated conclusively that c-Myc was sufficient to elicit apoptosis in primary rodent embryo fibroblasts (REFs) or established Rat1 fibroblasts deprived of growth factors. This study was based on resolution of the fulcrum observation that c-Myc was not able to drive outgrowth of a cell population cultured at suboptimal serum concentrations, even though in the same population c-Myc was clearly stimulating cell cycle transit. The reason behind this apparent discrepancy was that cell death and cell division was occurring simultaneously in different cells in the population. The cell death that was occurring had all the features of apoptosis. As in 32D cells apoptosis, occurred in all phases of the cell cycle, in a stochastic fashion such that some cells in the population died while other cells continued to proliferate through the influence of c-Myc. The stochastic feature, which was the basis for the initial apparent conundrum, remains an enduring mystery in apoptosis not only by c-Myc but generally. Notably, additional growth limiting treatments such as amino acid deprivation were similarly capable of eliciting apoptosis by c-Myc, raising the possibility that a conflict of growth and arrest signals in cells might be the cause of apoptosis rather than a direct function of c-Myc (Evan *et al.*, 1992). A rudimentary genetic analysis performed using a canonical set of c-Myc deletion mutants (Stone *et al.*, 1987) indicated overlap in the terminal regions of c-Myc required for apoptosis or transformation, illustrating crucial requirements for the Myc Box-containing NTD and the b/HLH/LZ-containing CTD (Evan *et al.*, 1992). Cell death by c-Myc in the Rat1 system was subsequently shown to be associated with the activation of certain Jun kinases (Jnks) and caspase-3 (Kagaya *et al.*, 1997; Yu *et al.*, 1997; Kangas *et al.*, 1998), which is crucial to produce the associated chromatin collapse and nucleosomal DNA degradation. c-Myc is also responsible for cell death in Burkitt's lymphoma cells and EBV-immortalized B cells deprived of autocrine factors (Milner *et al.*, 1993; Cherney *et al.*, 1994). Lastly, epithelial cells have also been shown to be susceptible to apoptosis by c-Myc (Sakamuro *et al.*, 1995). Malignant transformation of epithelia in lung, colon, breast, prostate, cervix and liver presents a well-known and grim clinical challenge. However, epithelial cells are not only susceptible to c-Myc but can be killed via multiple mechanisms, some of which appear to be unavailable in fibroblasts or hematopoietic cells (Sakamuro *et al.*, 1995; Lanoix *et al.*, 1996; Trudel *et al.*, 1997). Together these studies showed that c-Myc is a sufficient cause of apoptosis.

A necessary role for c-Myc in apoptosis was reported by Shi *et al.* (1992) who used antisense oligonucleotides to reduce c-Myc expression in murine

T hybridomas and show that c-Myc was required for apoptosis induced by T cell receptor activation. Later studies established that c-Myc was a critical determinant of apoptosis induced by TNF-α (Janicke *et al.*, 1994; Klefstrom *et al.*, 1994; Dong *et al.*, 1997) and of the magnitude of the response to ligation of the CD95/Fas death receptor (Hueber *et al.*, 1997). However, it now appears that c-Myc is required for efficient response to a variety of apoptotic stimuli, including transcription and translation inhibitors, hypoxia, glucose deprival, heat shock, chemotoxins, DNA damage, and cancer chemotherapeutics (Evan *et al.*, 1992; Harrington *et al.*, 1994a; Wagner *et al.*, 1994; Yao *et al.*, 1995; Alarcon *et al.*, 1996; Graeber *et al.*, 1996; Jiang *et al.*, 1996; Kang *et al.*, 1996; Li *et al.*, 1996; Dong *et al.*, 1997; Koumenis and Giaccia, 1997; Zhan *et al.*, 1997; Nesbit *et al.*, 1998; Rupnow *et al.*, 1998; Shim *et al.*, 1998). Evan and Littlewood have proposed the appealing idea that c-Myc does not act as a death effector in all these instances but instead acts to sensitize cells to a variety of apoptotic triggers (Evan and Littlewood, 1998). In any case, its role in death by so many stimuli supports the hypothesis that c-Myc has intrinsic function related to cell death.

Retroviral myc proteins (v-*myc* proteins) and other Myc family members also have the capacity to induce apoptosis. v-*myc* proteins mimic c-Myc despite the fact that they include a variety of sequence alterations which might have been thought to ablate apoptotic properties (Troppmair *et al.*, 1992; Wang *et al.*, 1993a,b; Dolnikov *et al.*, 1996). Indeed, a comparison of c-Myc and v-Myc in avian cells indicated that the latter induced apoptosis more potently (Petropoulos *et al.*, 1996). The tight association between these properties in retroviral oncogenic *myc* genes, where strong selective pressures would be expected to rid death-dealing aspects, reinforces the notion that they are inseparable at the level of the protein and therefore coordinately controlled together. Investigations of the capacity of N-Myc and L-Myc to drive cell death have revealed similarities to c-Myc (Zornig *et al.*, 1995; Ueda and Ganem, 1996; Chernova *et al.*, 1998; Lutz *et al.*, 1998; Nesbit *et al.*, 1998). The antiproliferative S-Myc protein also induces apoptosis but without the need for a growth factor deprivation trigger (Asai *et al.*, 1994). A comparison of the effects of N-Myc, L-Myc, and c-Myc in 32D myeloid cells indicates that they differentially sensitize cells to some stimuli (Nesbit *et al.*, 1998). The literature provides counter examples where under growth limiting conditions c-Myc can promote survival of B lymphocytes (Wu *et al.*, 1996a; Sonenshein, 1997; Wang *et al.*, 1999) or differentiation of normal human keratinocytes (Gandarillas and Watt, 1997), illustrating the complex cell fate regulating aspects of c-Myc. However, the clear trend in the great majority of cell types examined is for Myc proteins to promote both mitogenesis and cell death under growth limiting conditions.

The cooperation in tumorigenesis between c-Myc and Bcl-2 (Strasser *et al.*, 1990), a gene discovered at a chromosomal breakpoint in a follicular B cell tumor (Tsujimoto *et al.*, 1984) and then subsequently identified as an apoptosis suppressor that can cooperate with c-Myc in cell immortalization (Vaux *et al.*, 1988), made it likely that Bcl-2 would block apoptosis by c-Myc. Several studies have explicitly confirmed this expectation in mesenchymal cells (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993b; Wang *et al.*, 1993b). This work helped establish the principle that manifestation of the full oncogenic properties of c-Myc may require loss or suppression of apoptosis (Evan *et al.*, 1992; Facchini *et al.*, 1994). The ability of other oncogenes to collaborate with *myc* in lymphomagenesis, including *ras*, *raf*, *pim-1* and v-*abl*, suggests that these oncogenes can also provide as part of their action an antiapoptotic signal which negates the death-dealing aspect of c-Myc (Adams and Cory, 1992). How these genes, and in particular Bcl-2, impinge mechanistically on c-Myc remains unclear. For example, Bcl-2 does not block apoptosis by c-Myc in certain epithelial tissues (Trudel *et al.*, 1997). Moreover, caspase inhibitors do not block cell death by c-Myc (although they block apoptotic features associated with it) (McCarthy *et al.*, 1997), even though Bcl-2 family proteins are thought to block death by inhibiting activation of Apaf-1 and thereby caspases (Adams and Cory, 1998; Green and Reed, 1998; Reed *et al.*, 1998). Finally, it is important to note that Bcl-2 proteins act by affecting the kinetics of death in cells expressing c-Myc, which are certainly important in cancer cells, rather than by inhibiting signals for death commitment (McCarthy *et al.*, 1997), about which little seems to be known. Thus it seems that c-Myc and other proapoptotic oncogenes coordinately induce signals for death commitment and caspase activation. Emerging caspase-independent aspects of apoptosis (e.g. Quignon *et al.*, 1998; Wang *et al.*, 1998b) may give insights into the manner in which a death commitment signal is generated and into the exact relationship between c-Myc and mitochondrial events that are regulated by the Bcl-2 family.

p53: obligate effector or simply death sensitizer?

The role of p53 in apoptosis by many stimuli is well-established but its exact role in apoptosis by c-Myc is uncertain. Several *in vitro* studies have argued that p53 is crucial. Mouse embryo fibroblasts from p53 null animals are largely refractory to apoptosis by c-Myc (Hermeking and Eick, 1994; Wagner *et al.*, 1994), and similar results have been obtained in other mesenchymal cell types (Wang *et al.*, 1993a; Hermeking *et al.*, 1994; Bennett *et al.*, 1995; Yu *et al.*, 1997). Some investigators also observed elevation of p53 levels by ectopic c-Myc expression (Hermeking and Eick, 1994; Yu *et al.*, 1997), in support of the candidacy of p53 as a Myc target gene (Reisman *et al.*, 1993), although this effect has not been observed widely. However, results from other studies which included *in vivo* assays and epithelial cell models argued against an obligate role for p53 (Hsu *et al.*, 1995; Sakamuro *et al.*, 1995; Lenahan and Ozer, 1996; Trudel *et al.*, 1997). For example, even though c-Myc induces lymphomas more rapidly in p53 null mice (Elson *et al.*, 1995), a careful examination showed this was due to enhanced proliferation and not to reduced apoptosis (Hsu *et al.*, 1995). In addition, expression of SV40 T antigen, which inactivates both p53 and Rb, or adenovirus E1B 55 kD protein, which inactivates p53, did not affect the ability of c-Myc to induce apoptosis in established

Rat1 cells (Harrington *et al.*, 1994b; Lenahan and Ozer, 1996). Finally, overexpressed c-Myc induces apoptosis in M1 myeloid cells, which lack a p53 gene (Selvakumaran *et al.*, 1994b; Lotem and Sachs, 1995). Tissue specific differences may dictate the mechanisms used by c-Myc. In baby rat kidney (BRK) cells, c-Myc induces apoptosis by both p53-dependent or p53-independent mechanisms (Sakamuro *et al.*, 1995). The lack of a p53 requirement for apoptosis was confirmed in kidney epithelia in *myc* transgenic mice (Trudel *et al.*, 1997). It is interesting to note in this context that p53 inactivation does not cooperate with c-Myc to promote malignant transformation of mammary epithelial tissues (Elson *et al.*, 1995). Thus, p53-independent mechanisms may be predominant in epithelial cells. A complex relationship between c-Myc and p53 is suggested by the following observations. Interestingly, mutant p53 suppresses apoptosis by c-Myc in M1 myeloid cells, even though they are nullizygous for p53 (Lotem and Sachs, 1995). This effect is presumably dictated by the ability of mutant p53 to interact directly or indirectly with a factor required by c-Myc to induce death. The results of a study examining the effects of c-Myc on TNF killing confirm some type of crosstalk between c-Myc and p53. In this study, it was noted that p53 null cells were refractory to c-Myc sensitization to TNF, but this phenotype could not be replicated by overexpression of a C-terminal dominant inhibitory fragment of p53 (Klefstrom *et al.*, 1997). Thus, the presence of unoligomerized wild-type p53, which would be expected to be transcriptionally inactive, was sufficient to allow c-Myc sensitization. Thus, some transactivation-independent aspect of p53 is crucial. One interpretation of these data is that c-Myc and p53 interact with some common apoptosis effector signaling system, perhaps involving the apoptosis effector domain in p53, as we have speculated previously (Sakamuro *et al.*, 1997). This idea is supported by a recent study of the p53-interacting modulator $p33^{ING1}$ in cells where apoptosis is induced by c-Myc, which suggests that p53 and c-Myc may have parallel actions (Helbing *et al.*, 1997). In any case, it is conceivable that p53 or c-Myc may sensitize cells to each other or to other proapoptotic triggers, an idea related to that proposed by Evan and Littlewood (1998) to explain the proapoptotic actions of oncogenes. Further investigations are needed to understand what is apparently a complex relationship between c-Myc and p53.

$p19^{ARF}$, the alternate reading frame product of the Ink4 tumor suppressor gene, has been implicated recently as a link between p53 and c-Myc in apoptosis (Zindy *et al.*, 1998). Ink4 is among the most frequently mutated tumor suppressor genes in human cancer. It encodes $p16^{INK4}$, a cell cycle kinase inhibitor (CKI) that is crucial for proper regulation of the retinoblastoma (Rb) protein, as well as $p19^{ARF}$, a protein that interacts with and regulates the p53-Mdm2 complex (Sherr, 1998). Thus, the Ink4 gene encodes two structurally unrelated tumor suppressors that interact with the two most important growth regulatory circuits in the cell. It was noted that mouse embryo fibroblasts (MEFs) lacking the $p19^{ARF}$ gene could be transformed by oncogenic Ras alone, suggesting that c-Myc might regulate $p19^{ARF}$ (Zindy *et al.*, 1998). In support of this hypothesis, introduction of c-Myc into wild-type MEFs elevated $p19^{ARF}$ and p53 and triggered crisis and apoptosis, whereas c-Myc had little effect on p53 or apoptosis in $p19^{ARF-/-}$ MEFs. Wild-type MEFs transfected with c-Myc that emerged as immortal clones invariably sustained mutation of p53 or deletion of $p19^{ARF}$. $p19^{ARF-/-}$ and $p53^{-/-}$ MEFs were readily immortalized by c-Myc but exhibited an attenuated death response to serum deprival (Zindy *et al.*, 1998). Following a wave of apoptosis which eliminated a large fraction of the cell population, the remaining cells could be passaged indefinitely in the absence of serum. The initial wave of apoptosis apparently reflects a $p19^{ARF}$/p53-independent mechanism of the type discussed above. Nevertheless, loss of p53 or $p19^{ARF}$ tolerized a significant portion of the cell population to the cytotoxic effects of c-Myc. $p19^{ARF}$ is expressed from a specific promoter in the Ink4 gene that includes Myc-like E box motifs (C Sherr, personal communication), making it a logical target gene candidate. Whether $p19^{ARF}$ plays a similar role for c-Myc in other cell types and organisms is not yet clear. Rat1 fibroblasts appear to have both intact wild-type p53 and $p19^{ARF}$ genes (J Sedivy, personal communication), which may explain why they are so sensitive to apoptosis by c-Myc, but it is notable that neither gene was mutated during establishment of these cells. In MEFs, $p19^{ARF}$ responds similarly to ectopic expression of E1A and provides a link to Mdm2 through E1A mediates p53 stabilization (de Stanchina *et al.*, 1998). How E1A induces $p19^{ARF}$ is unclear. Zindy *et al.* (1998) proposed that $p19^{ARF}$ regulates a p53-dependent checkpoint that safeguards cells against hyperproliferative and oncogenic signals. That $p19^{ARF}$ is dispensable for cell proliferation and possibly a c-Myc target gene specifically involved in apoptosis impacts the dual signal model for c-Myc function considered below.

Myc target genes and apoptosis

A growing number of genes have been identified as targets for regulation by c-Myc and it seems likely that more will follow (Dang, 1999). Two recent studies raise concerns about physiological validation (Bush *et al.*, 1998; Xiao *et al.*, 1998). Using c-*myc* null cells (Mateyak *et al.*, 1997) to assess the serum response of the best-studied target genes, it was found that only the *cad* and *gadd45* genes were misregulated in the absence of c-Myc (Bush *et al.*, 1998). *cad* is a housekeeping gene that participates in pyrimidine biosynthesis which is activated by c-Myc (Miltenberger *et al.*, 1995; Boyd *et al.*, 1998), but it has not been assigned any role in apoptosis to date. *gadd45* (growth arrest and DNA damage-inducible gene 45) is functionally undefined and repressed by c-Myc (Marhin *et al.*, 1997); it also has not been assigned any role in cell death. It is notable that of the >30 candidate genes identified to date, few seem as robustly regulated as the targets of better understood transcription factors. Although biochemical crosslinking methodologies suggest that there may be many c-Myc binding sites in the genome (Boyd *et al.*, 1998), recent cell biological experiments suggest the number of physiological sites may be relatively small (Rudolph

et al., 1998). In these experiments, primary REFs were transfected with multimerized wild-type physiological DNA binding sites or point-mutated versions of the same and cell clones were selected and monitored for the number of passages to arrive at replicative crisis. Interestingly, clones containing as few as ~50 copies of the wild-type E box sequences underwent crisis significantly more quickly than controls (Rudolph *et al.*, 1998). If, as expected, these sequences compete with physiological sites for binding to c-Myc, then these results imply that the number of loci needed for c-Myc to drive proliferation are comparatively few. If so, many genes which score as c-Myc targets may be fortuitously rather and physiologically regulated.

That caveat stated, there is evidence that some of the existing targets indeed mediate some of the biological effects of c-Myc, and several linked to apoptosis are surveyed here. Ornithine decarboxylase (ODC) is perhaps the best studied target of c-Myc (Bello-Fernandez *et al.*, 1993; Wagner *et al.*, 1993a; Tobias *et al.*, 1995; Wu *et al.*, 1996b; Packham and Cleveland, 1997; Ben-Yosef *et al.*, 1998). Significantly, ODC has been shown to be necessary and sufficient for apoptosis by c-Myc in 32D myeloid cells (Packham and Cleveland, 1994). ODC does not drive cell death nearly as efficiently as c-Myc but in support of its role it has been reported to be an important participant with c-Myc in chemotoxin-induced cell death (Zhan *et al.*, 1997). ODC is a housekeeping enzyme involved in polyamine synthesis and is necessary for cell proliferation. How it impacts apoptosis is unclear, although one possibility is that excess polyamine catabolism generates reactive oxygen species (ROS) which promote mitochondria-dependent apoptosis (Packham and Cleveland, 1995). ODC has overlapping roles in death and proliferation (Auvinen *et al.*, 1992; Moshier *et al.*, 1993; Packham and Cleveland, 1994; Shantz and Pegg, 1994).

Several genes that promote cell cycle transit have been suggested as c-Myc targets (Dang, 1999) and two of these, cyclin A and Cdc25A, have been suggested to participate in apoptosis by c-Myc (Hoang *et al.*, 1994; Galaktionov *et al.*, 1996). Integrins regulate cyclin A, which associates with CDK2 and promotes S phase progression. Enforced expression of cyclin A in Rat1 cells is sufficient to confer anchorage-independent growth capacity and susceptibility to apoptosis by serum deprival (Hoang *et al.*, 1994). Cdc25A is a phosphatase responsible for activating cdc2. Cdc25A has been reported to be both necessary and sufficient for apoptosis by c-Myc (Galaktionov *et al.*, 1996). Although this observation has not been extended, it is consistent with suggestions of a role in apoptosis in particular settings for aberrant activation of cdc2 (also a putative target gene [Born *et al.*, 1994]) (Shi *et al.*, 1994; Chen *et al.*, 1995). Three other cell cycle regulators that are not genetic targets of c-Myc, Cdk2, Cdk3, and cyclin D3, have also been reported to enhance apoptosis by c-Myc (Janicke *et al.*, 1996; Braun *et al.*, 1998). Early ideas that apoptosis might have features of inappropriate mitosis stimulated investigations of cell cycle-related genes in apoptosis. However, these ideas have been challenged recently because positive associations with death have not been widely generalized. One study that employed a variety of cell cycle inhibitors concluded that Cdks are dispensable for apoptosis (Rudolph *et al.*, 1996). In addition, a more recent study noted that Cdk2 and Cdc25A message levels reacted similarly in Rat1 cells, whether they underwent apoptosis in the presence of deregulated c-Myc or simply exit to G0 in its absence (Helbing *et al.*, 1998). The status of Cdks in apoptosis by c-Myc deserves further attention but is uncertain at this juncture.

As noted above, $p19^{ARF}$ is probably a transcriptional target of c-Myc (Dang, 1999), because the $p19^{ARF}$ message is initiated at a specific promoter in the Ink4 gene that includes Myc E box motifs (C Sherr, personal communication). $p19^{ARF}$ is dispensable for cell proliferation so it will likely have a specific role either in priming death or in mediating death or death sensitization signals. Since it is the only target gene at current which appears to be strictly devoted to apoptosis, analysis of the mechanisms by which c-Myc activates its expression may provide a physiological paradigm for death-specific transactivation. Other proapoptotic oncogenes are likely to upregulate $p19^{ARF}$ and it will be important to learn whether c-Myc mediates such effects. Continued investigations of the c-Myc-$p19^{ARF}$ connection and the regulation and mechanism of action of $p19^{ARF}$ should provide interesting and important new insights into one pathway through which c-Myc kills cells.

The metabolic enzyme lactate dehydrogenase A (Ldh-A) is a c-Myc target gene recently shown to sensitize cells to a novel glucose-dependent apoptotic pathway (Shim *et al.*, 1997, 1998). Ldh-A is part of the normal anaerobic glycolysis pathway which operates at higher levels in hypoxic cells. In tumor microenvironments, cells further than ~0.1 mm from a blood supply are subjected to severe hypoxia which they must adapt to or perish. In addition to recruiting vessels by secreting angiogenic factors, cells adapt to hypoxic conditions by metabolizing glucose without oxygen and overproducing lactic acid aerobically, in a process known as the Warburg effect (Warburg, 1956; Dang, 1999). Notably, MCF7 breast carcinoma cells deprived of glucose exhibit both c-Myc elevation and significant cell death, which can be blocked by the addition of antisense c-Myc oligonucleotides (Lee *et al.*, 1997). The identification of Ldh-A as a genetic target of c-Myc may provide a molecular basis for this effect (Shim *et al.*, 1997). Notably, when Rat1 cells overexpressing Ldh-A are deprived of glucose or are treated with the antimetabolite 2-deoxyglucose, they engage a p53-independent death program, whereas control cell lines merely respond by G0/G1 arrest (Shim *et al.*, 1998). c-Myc-expressing Rat1 cells also respond to glucose deprivation or 2-deoxyglucose treatment by undergoing apoptosis. The extent of apoptosis in cells expressing either c-Myc or Ldh-A is similar. Ldh-A-expressing cells do not undergo apoptosis following serum deprival, indicating that Ldh-A only sensitize cells to the antiglycotic trigger, but the extent of apoptosis caused by glucose deprival is similar to that caused in c-Myc-expressing cells by serum deprival (Shim *et al.*, 1998). Bcl-2 suppresses glucose deprival-induced apoptosis in both c-Myc-expressing Rat1 cells and MCF7 carcinoma cells (Lee *et al.*, 1997; Shim *et al.*, 1998). Therapeutic opportunities are suggested by the ability of other frank tumor cells which overexpress c-Myc to undergo

significant apoptosis when treated with 2-deoxyglucose (Shim *et al.*, 1998). The mechanism of action is unclear at this point but is speculated to involve alteration of the redox state of the cell due to relative increases in the ratio of NAD+ : NADH caused by Ldh-A-dependent generation of NAD+ (Shim *et al.*, 1998).

Regulation by cytokines and adhesion signals

The apoptotic properties of c-Myc are masked by cytokines and adhesion signals. The role of cytokines was obvious since it was removal of such factors that revealed those properties. An examination of the inhibitory effects of different serum growth factors in Rat1 fibroblasts identified insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) as crucial players (Harrington *et al.*, 1994a). Insulin-like growth factor-II (IGF-II) also had some antiapoptotic activity but it was much weaker. Interestingly, experiments where protein synthesis inhibitors were added indicated that PDGF required protein synthesis to mediate its effects but that IGF-1 did not. Bcl-2 upregulation by PDGF was ruled out and the presumptive genetic targets of PDGF remain to be identified. In contrast, IGF-1 apparently regulates cell death at some posttranslational level. Cell type differences may be a caveat because in one study in hepatoma cells IGF-1 seemed to promote apoptosis by c-Myc (Xu *et al.*, 1997). However, antiapoptotic effects of IGF-1 are clearly of wide significance in cancer settings (Baserga *et al.*, 1997). IGF-1 has many roles in cell growth and differentiation but survival signals that are mediated by the IGF-1 receptor are mechanistically distinct (O'Connor *et al.*, 1997). TGF-α also appears to suppress apoptosis by c-Myc, in this case during development of mammary carcinoma in transgenic mice (Amundadottir *et al.*, 1996). Tissue specific differences are again hinted at because TGF-α mediates its effects through the EGF receptor, but EGF was not found to promote survival in Rat1 cells (Harrington *et al.*, 1994a). Lastly, activation of the tyrosine kinase receptor Ark (Axl) by its ligand Gas6 (a member of the vitamin K-dependent family of proteins preferentially expressed in quiescent cells) has been reported to suppress apoptosis by c-Myc (Bellosta *et al.*, 1997). No mechanistic information is available concerning the signaling pathways used by TGF-α or Gas6/ARK.

The IGF-1 survival signal implicated by Harrington and colleagues has been traced from the IGF-1 receptor through a Ras-dependent pathway to phosphatidylinositol 3′-kinase (PI3′K) and the AKT/PKB kinase (Kauffman-Zeh *et al.*, 1997). The PI3′K-AKT pathway has been widely implicated in cell survival mediated by cytokine and cell adhesion receptors, with Ras having an important part in mediating the signal from cytokine receptors (Frisch and Ruoslahti, 1997; Downward, 1998). At least two AKT substrates are part of the basic apoptosis machinery, Bad and caspase-9, whose proapoptotic activity is each inhibited by AKT phosphorylation (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998). Caspase-9 was implicated recently in the mechanism through which c-Myc potentiates cell death (Fearnhead *et al.*, 1998), so the antiapoptotic effects of IGF-1 might lead here. However, the extent of any role for caspase-9 inhibition in mediating the antiapoptotic effects of IGF-1 remains to be established and it seems reasonable to expect that other substrates will also be important. As noted above, cyclin D3 can potentiate death by c-Myc (Janicke *et al.*, 1996) and recently this cyclin has been reported to be a substrate for AKT (Muise-Helmericks *et al.*, 1998). It is also conceivable that other Ras effectors may also be involved in mediating IGF-1-generated survival signals, such as those involving Rho or NF-κB (Lebowitz *et al.*, 1995; Mayo *et al.*, 1997). Lastly, a recent study has implicated CD95/Fas in death by c-Myc (see below) and AKT has been reported to inhibit CD95/Fas-dependent death in this context (Rohn *et al.*, 1998). In summary, the manner in which IGF-1 inhibits apoptosis by c-Myc may prove complex and involve several antiapoptotic pathways.

Cell adhesion also influences the susceptibility of cells to apoptosis by c-Myc. In a study of CHO cells overexpressing c-Myc, cell density was found to alter the susceptibility to death triggered by serum deprivation (Gibson *et al.*, 1995). Cells cultured at higher densities were less susceptible, arguing that cell-cell interactions may limit death. This observation is reminiscent of density-dependent effects on anoikis in MDCK cells (Frisch and Francis, 1994). Cell-substratum interactions also influence susceptibility to c-Myc. For example, primary rat embryo fibroblasts (REFs) transformed by c-Myc and oncogenic Ras will rapidly undergo apoptosis if deprived of matrix adhesion, whereas normal REFs exit to G0 phase for an extended period (McGill *et al.*, 1997). RGD-containing integrin ligands also induced apoptosis of such cells, implying that integrin signals are responsible for death suppression. Death was inhibited if cells are plated at high density under conditions which promote cell-cell aggregation, which was implicated to be via cadherins since calcium chelators relieved inhibition. Loss of wild-type p53 lengthened the kinetics but did not abrogate death, consistent with the notion that p53 sensitizes cells to apoptosis c-Myc but is not required. Lastly, REFs cotransformed with activated Src instead of Ras did not undergo apoptosis when deprived of adhesion, probably due to the role of Src in integrin signaling (Inoue *et al.*, 1995; Frisch and Ruoslahti, 1997; Parsons and Parsons, 1997).

Another study using primary chick embryo fibroblasts (CEFs) also implicated matrix signals in the susceptibility to apoptosis by c-Myc (Crouch *et al.*, 1996). In this study, fibronectin and collagen matrix reduced apoptosis caused by serum withdrawal, and substratum coated with an anti-β1 integrin antibody produced a similar effect. Consistent with a role for integrin signaling in governing susceptibility to c-Myc, proteolysis of the integrin-associated effector kinase FAK was observed to be an early event in cells committed to undergo apoptosis (Crouch *et al.*, 1996). Adhesion signals involving Src and APC/β-catenin regulate both apoptosis and c-Myc expression (Barone and Courtneidge, 1995; He *et al.*, 1998), consistent with the possibility of some role for an adhesion-dependent signal in modulating the ability of c-Myc to activate cell death. Further investigation is needed to assess whether loss of integrin-mediated

adhesion is a triggering event or a correlate of apoptosis by c-Myc. In addition, given the findings of these studies, it would be interesting to determine whether ectopic expression of activated Src or an uncleavable FAK construct could block apoptosis by c-Myc which is elicited by serum or matrix deprival. One might predict an overlap in the processes used by IGF-1 and integrins to suppress the lethality of c-Myc, insofar as there is crosstalk between the IGF-1 receptor and integrin circuitry [e.g. $\alpha_v\beta_3$ (Zheng and Clemmons, 1998) and $\beta 1$ receptors (Guilherme and Czech, 1998). Moreover, integrins also access the PI3′AKT signal transduction pathway to promote cell survival (Frisch and Ruoslahti, 1997; Clark *et al.*, 1998; Kumar, 1998).

Regulatory connections between c-Myc and death receptors

Several studies reveal regulatory circuits that link c-Myc to death receptors (see Figure 3). Members of the tumor necrosis factor (TNF) or APO transmembrane receptor family are expressed on the surface of many mesenchymal and epithelial cells. They have complex roles that extend beyond death signaling including possible roles in proliferation (e.g. Zornig *et al.*, 1998). TNF family receptors use an adaptor system to directly activate caspases and they are subject to complex modulation (Ashkenazi and Dixit, 1998). Ligand binding causes receptor homotrimerization and recruitment on the cytoplasmic face of the membrane of a death-inducing signaling complex (DISC), which includes the adaptors FADD and TRADD and procaspase-8 as crucial physiological death effectors (Juo *et al.*, 1998; Varfolomeev *et al.*, 1998; Yeh *et al.*, 1998; Zhang *et al.*, 1998). A brief outline of the CD95/Fas and TNf receptor systems follows since c-Myc has been implied to act both upstream and downstream of each (Janicke *et al.*, 1994; Klefstrom *et al.*, 1994, 1997; Hueber *et al.*, 1997; Wang *et al.*, 1998a).

CD95/Fas activates death by two routes that are either independent or dependent of mitochondria, as favored in Type I or Type II cells, respectively (Scaffidi *et al.*, 1998). In type I cells, recruitment of FADD leads to efficient binding and activation of caspase-8 activity, which proceeds to activate caspase-3 and cause cell demise. In type II cells, caspase-3 is activated by a more roundabout route that involves mitochondria. In this case, activation of caspase-8 occurs with delayed kinetics and is weaker. With time the proapoptotic Bcl-2 family protein Bid is cleaved and activated by the active caspase-8 which is produced, and active Bid proceeds to induce cytochrome c release and caspase-9-dependent activation of caspase-3 (Li *et al.*, 1998; Luo *et al.*, 1998). Antiapoptotic Bcl-2 proteins inhibit CD95/Fas-dependent apoptosis in Type II cells, because of the involvement of mitochondria, but not in Type I cells, since mitochondria are not significantly involved. CD95/Fas also activates the Jnk pathway but there is conflicting evidence regarding how. In one study where caspase-8 was genetically ablated, Jnk activation by CD95/Fas was defective (Juo *et al.*, 1998). However, other studies imply activation occurs upstream or in parallel to FADD (Wajant *et al.*, 1998), possibly through the putative CD95/Fas-interacting adaptor protein Daxx, which is proposed to activate the Jnk pathway through binding the Mekk1 kinase Ask1 (Yang *et al.*, 1997; Chang *et al.*, 1998). These interactions await physiological confirmation as do those with other factors reported to modulate CD95/Fas in model systems, including the proapoptotic adaptor protein Faf and the antiapoptotic ubiquitin-like protein sentrin (Chu *et al.*, 1995; Okura *et al.*, 1997). The TNF-R also uses FADD and caspase-8 to activate cell death, but its DISC includes the other adaptor proteins TRADD, RIP, and the TRAFs (Ashkenazi and Dixit, 1998). TRADD is a proapoptotic intermediary adaptor between the receptor and FADD. RIP also binds TRADD and is thought to participate in activation of the Jnk pathway, which also involves TRAFs and possibly Ask1 (Ashkenazi and Dixit, 1998; Nishitoh *et al.*, 1998). TRAFs, in particular TRAF2, are implicated in activation of an antiapoptotic pathway that stimulates NFκB activation via IκB inactivation (Ashkenazi and Dixit, 1998).

The initial connection between c-Myc and death receptors was suggested by findings that c-Myc is a crucial determinant of the cytotoxic response to TNF-α (Janicke *et al.*, 1994; Klefstrom *et al.*, 1994). Subsequent work on the mechanism has argued that c-Myc acts by influencing the delicate balance between the survival and death signaling pathways which are simultaneously activated by this factor (Klefstrom *et al.*, 1997). Using a conditional Rat1 expression system, these authors showed that induction of c-Myc impaired the ability of TNF-α to activate NF-κB and Jnk. The inhibition of NF-κB was implied to be important because ectopic expression of NF-κB made cells refractory to the ability of c-Myc to sensitize them to TNF-α. While this study did not examine a role for Jnk, nor did it establish if NF-κB inactivation was necessary for the effect of c-Myc (only that it was sufficient to block it), these results infer that c-Myc may sensitize cells to TNF-α by impeding the TRAF2-dependent survival pathway which is thought to be responsible for coactivation of NF-κB and Jnk (Natoli *et al.*, 1997; Reinhard *et al.*, 1997). It will be important to explicitly establish TRAF2 involvement, however, because the ability of TNF-α to still activate NF-κB in TRAF2-deficient cells implies there are other ways to do it (Yeh *et al.*, 1997). Notably, c-Myc did not block TNF-α-dependent elevation of p53 which occurred, indicating that c-Myc only affected a particular signaling pathway(s) not involved in activation of TNF-α target genes (Klefstrom *et al.*, 1997). Interestingly, as noted above, a transactivation-independent function of p53 was implied to be necessary for c-Myc sensitization, because inhibiting p53 oligomerization by a dominant inhibitory approach did not mimic the resistance seen in p53 null cells. Taken together, the results hinted that c-Myc acts at a step proximal to TRAF2 which regulate TNF-α-dependent activation of NF-κB and Jnk. Further investigation is required to determine the exact mechanism and the apparent regulatory linkage with the TNF receptor.

c-Myc may act both upstream and downstream of the TNF-R family member CD95/Fas (Hueber *et al.*, 1997). Similar to the case with TNF-α, Rat1 fibroblasts were not susceptible to killing by CD95/Fas ligand unless c-Myc was expressed. This indicated that c-Myc

was necessary or perhaps that it sensitized cells to this factor (Evan and Littlewood, 1998). The latter possibility is supported by evidence that transcription of the CD95/Fas ligand is targeted for activation by many apoptotic stimuli, including TCR activation through NF-κB (Kasibhatla *et al.*, 1999), DNA damage and other stresses through NF-κB, the Jnk pathway (AP-1), and Egr-3 (Faris *et al.*, 1998; Kasibhatla *et al.*, 1998; Mittelstadt and Ashwell, 1998). Sensitization by c-Myc in Rat1 fibroblasts was not due to upregulation of CD95/Fas or CD95/Fas ligand, although recent evidence suggests that CD95/Fas ligand may be a target for activation by c-Myc in other cell types (Wang *et al.*, 1998a). In any case, the biological data indicates that c-Myc can sensitize cells to CD95/Fas ligand by acting at some point downstream of the receptor.

Evidence was also presented in support of the hypothesis that CD95/Fas is necessary for apoptosis by c-Myc elicited by serum deprival (Hueber *et al.*, 1997). First, neutralizing antibodies to the CD95/Fas ligand inhibited death in a dose-dependent manner when added to serum deprived cells. Consistent with a specific role in apoptosis, the antibodies did not affect the ability of c-Myc to drive cell division in the absence of serum. Second, embryo fibroblasts from *lpr* or *gld* mice which have defective CD95/Fas and CD95/Fas ligand genes, respectively, were refractory to apoptosis by c-Myc. Finally, ectopic expression of a FADD dominant inhibitory mutant also inhibited the cytotoxic effects of c-Myc. These results all pointed to an effector role for CD95/Fas in c-Myc killing in Rat1 fibroblasts. A subsequent study aimed at identifying where c-Myc acted traced the point downstream of ligand binding but actually upstream of FADD binding (Rohn *et al.*, 1998). A FADD-independent mechanism is also implied by the ability of overexpressed c-Myc to kill FADD nullizygous mouse embryo fibroblasts (Yeh *et al.*, 1998). FADD is the only physiologically validated effector of CD95/Fas so if it is dispensable some other proapoptotic CD95/Fas-binding adaptor protein may be involved, perhaps Daxx or Faf. A strong prediction of the hypothesis that CD95/Fas is crucial for apoptosis by c-Myc is that tumor development in *myc* transgenic animals should proceed more rapidly in *lpr* and *gld* backgrounds and in caspase-$8^{+/-}$ mice as compared with caspase-$3^{+/-}$ or caspase-$9^{+/-}$ mice (which would be predicted to be susceptible based on the finding that caspase-9 is responsible for mediating the proapoptotic action of c-Myc in cell free-extracts [Fearnhead *et al.*, 1998]). Further investigations are required to validate the role of CD95/Fas and to uncover the basis for its apparent regulatory interactions with c-Myc. Along with progress in understanding the connections to TNF receptor, studies of the CD95/Fas-c-Myc linkage seem

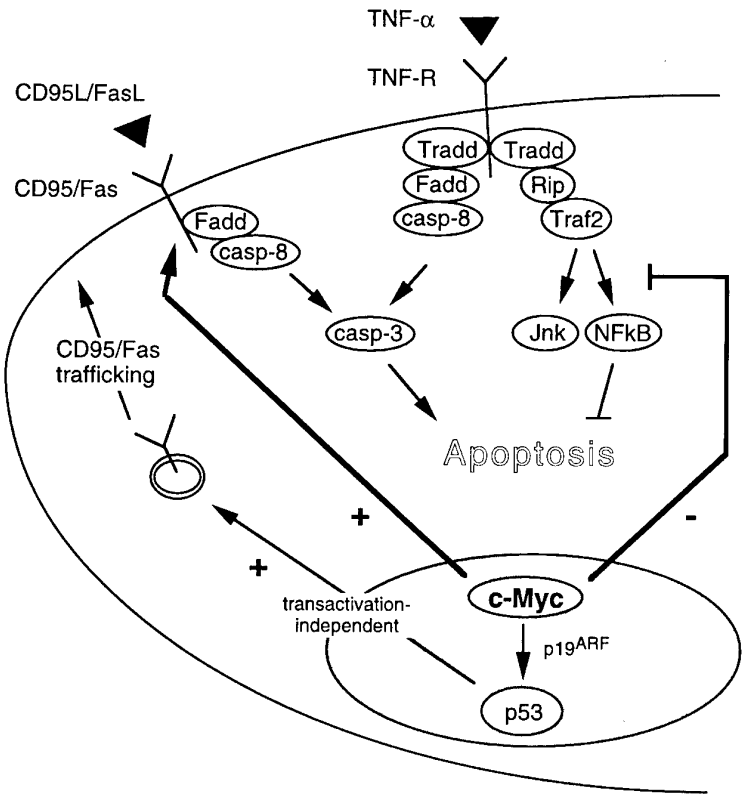


Figure 3 Regulatory links between c-Myc and death receptors. The cartoon outlines regulatory connections implicated between c-Myc and the death receptors TNF-R and CD95/Fas. c-Myc may sensitize cells to CD95/Fas in two ways, by promoting trafficking of internal stores of the receptor through a transactivation-independent action of p53, and by promoting the transmission of the CD95/Fas ligand signal at an undefined level downstream of the receptor and upstream of FADD (Hueber *et al.*, 1997; Rohn *et al.*, 1998). c-Myc may sensitize cells to TNF-α by inhibiting RIP/TRAF2-dependent antiapoptotic pathways that lead to activation of NF-κB and Jnk (Klefstrom *et al.*, 1997). How widely these connections are used in different cells is not yet clear

likely to give deeper insights into how c-Myc activates or sensitizes cells to apoptosis.

Role of interaction with the cell fate adaptor protein Bin1

Recent work from our laboratory suggests that interaction with the NTD-interacting adaptor protein Bin1 is required for c-Myc to activate apoptosis but not to drive cell proliferation (D Sakamuro, J DuHadaway, D Ewert and GC Prendergast, manuscript submitted). Bin1 was initially identified in a screen for MB1-binding proteins but its interaction with c-Myc requires both of the conserved Myc boxes (Sakamuro *et al.*, 1996). Bin1 inhibits the oncogenic and transcriptional transactivation properties of c-Myc in a binding domain-dependent manner (Elliott *et al.*, 1999; Sakamuro *et al.*, 1996). Bin1 will also inhibit the oncogenic properties of adenovirus E1A, papilloma virus E7, and mutant p53, through domains that are dispensable for effects on c-Myc (Elliott *et al.*, 1999). Investigations of the status of the Bin1 gene (Wechsler-Reya *et al.*, 1997b) in tumor cells supports the hypothesis that Bin1 is a tumor suppressor with important growth regulatory roles (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997b; Elliott *et al.*, 1999; Mao *et al.*, 1999; J DuHadaway, K Ge and GC Prendergast, manuscripts submitted).

In primary chick fibroblasts, which are susceptible to both apoptosis and malignant transformation by deregulated c-Myc alone, inhibition of Bin1 by antisense and dominant inhibitory methods suppressed serum deprival-induced apoptosis but not proliferation or malignant transformation induced by c-Myc. In particular, overexpression of the c-Myc binding domain of Bin1 inhibited apoptosis, implying that c-Myc-Bin1 interactions are required to mediate cell death. Similar results were obtained in BRK epithelial cells under conditions where p53-independent cell death by c-Myc was induced, arguing that Bin1 acts through a p53-independent mechanism. In Rat1 fibroblasts, Bin1 suppression inhibited apoptosis to a similar degree as Bcl-2 and allowed outgrowth of cells cultured in suboptimal serum conditions (Sakamuro *et al.*, manuscript submitted). Under conditions where Bin1 inhibition blocked apoptosis by c-Myc, no effects were seen on the message levels of three Myc target genes implicated in apoptosis (ODC, Cdc25A, and $p19^{ARF}$). Bin1 includes structural features found in signaling proteins (i.e. SH3 domain) so it may act to trigger apoptosis in a transcription-independent manner. Alternately, since Bin1 can inhibit the transactivation properties of c-Myc in transient assays (Elliott *et al.*, 1999b), there may be appropriate regulation of an unidentified target gene(s) relevant to apoptosis. Overexpression of Bin1 blocks transformation by c-Myc+Ras apparently by promoting the ability of c-Myc to drive death (unpublished observations). Bin1 overexpression kills tumor cells lacking endogenous Bin1 but it does not kill normal cells (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1998; Elliott *et al.*, 1999a), arguing that it is not an executioner but an adaptor protein that links c-Myc to the executioner machinery. Consistent with some regulatory role in apoptosis, Bin1 moves between the nucleus and cytosol and it is a genetic target for transcriptional regulation by NF-κB (Mao *et al.*, 1999). The relative ease with which one can obtain c-Myc-overexpressing cell clones that are resistant to apoptosis *in vitro* (Dhanaraj *et al.*, 1996) is consistent with observations that Bin1 is often inactivated in tumor cells by epigenetic mechanisms, which are relatively malleable (K Ge, J DuHadaway, D Sakamuro and GCP Prendergast, manuscripts submitted). Interestingly, Bin1 induces apoptosis in tumor cells which lack endogenous Bin1 through a largely caspase-independent mechanism that is not associated with chromatin collapse or nucleosomal DNA degradation (K Elliot, K Ge and GC Prendergast, manuscript submitted), features reminiscent of the death produced by c-Myc in the presence of caspase inhibitors (McCarthy *et al.*, 1997).

Existing information about Bin1 suggests that it is a cell fate adaptor protein that coordinates the complex decisions made when cells exit the cell cycle (e.g. enter G0, commit to die, differentiate, etc.). For example, in cells where c-Myc is normal and can be downregulated when there is a signal to exit the cell cycle, Bin1 helps to inhibit proliferation and to promote differentiation (Wechsler-Reya *et al.*, 1998; Mao *et al.*, 1999). In contrast, if c-Myc expression is enforced and as a result cells cannot exit the cell division cycle, Bin1 is necessary for c-Myc to engage the ensuing apoptotic program (Sakamuro *et al.*, manuscript submitted). Several findings argue for complex adaptor roles in cells for Bin1 (Bridging INtegrator-1). First, the nuclear tyrosine kinase c-Abl was shown recently to interact with but not phosphorylate Bin1 *in vivo* (Kadlec and Pendergast, 1997). c-Abl binding is mediated by the SH3 domain of Bin1 (Kadlec and Pendergast, 1997), which is dispensable for its association with c-Myc (Sakamuro *et al.*, 1996; Elliott *et al.*, 1999). A fraction of c-Abl in cells is reported to be activated at focal adhesions where integrins are located (Lewis *et al.*, 1996; Taagepera *et al.*, 1998), so since apoptosis by c-Myc is influenced by integrin signals (Crouch *et al.*, 1996) there may be links via this route to c-Abl activation. How Bin1 and c-Abl influence the action of each other in proliferation, differentiation, and apoptosis remains to be determined, however. Second, Bin1 is subjected to extensive alternate splicing, especially in neurons (Butler *et al.*, 1997; Ramjaun *et al.*, 1997; Tsutsui *et al.*, 1997; Wechsler-Reya *et al.*, 1997b; Ramjaun and McPherson, 1998), and it is localized to the cytosol as well as the nucleus in certain cells (Butler *et al.*, 1997; Kadlec and Pendergast, 1997; Wechsler-Reya *et al.*, 1998). The N and C-terminal regions of Bin1 are related to amphiphysin, a neuronal protein that is a paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.*, 1994; Dropcho, 1996). The same regions are also related to Rvs167 and Rvs161, two negative regulators of the cell cycle in yeast (Crouzet *et al.*, 1991; Bauer *et al.*, 1993) [Bin1 is related but not homologous since it cannot complement Rvs functions (Sakamuro *et al.*, 1996)]. Amphiphysin and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin isoform, have been implicated in receptor-mediated endocytosis (David *et al.*, 1996; Wigge *et al.*, 1997; Owen *et al.*, 1998). RVS167 and RVS161 have been implicated in endocytosis and karyogamy (Munn *et al.*, 1995; Brizzio *et al.*, 1998).

However, non-neuronal splice forms of Bin1 appear unlikely at this point to be involved in endocytosis, because only neuronal splice forms include the exons which encode clathrin-binding determinants needed for localization to endocytotic vesicles (Ramjaun and McPherson, 1998). The endocytosis connection in neurons might reflect a link in those cells between cell survival and the achievement of a differential and synaptically active state, which would be associated with neurotransmitter release and hence membrane trafficking. If Bin1 has endocytotic roles outside neurons, given the links of c-Myc to CD95/Fas and TNF-R, it is tempting to speculate about connections to mechanisms used by p53 to control apoptosis by cell surface trafficking of CD95/Fas (Bennett *et al.*, 1998), or to potential requirements of CD95/Fas internalization via endocytosis for the generation of a competent death signal (A Ashkenazi, personal communication). Third, the orthologs of Bin1 in yeast can influence cytoskeletal regulation and interact with a functionally undefined transmembrane protein (Sivadon *et al.*, 1995, 1997; Breton and Aigle, 1998). These roles hint of links to cell surface receptors and perhaps also to actin cytoskeletal rearrangements that are involved in membrane blebbing (zeosis), a process which has been reported to involve integrins, actin depolymerization, and Jnk activation events (Laster and Mackenzie, 1996; Huot *et al.*, 1998). Further investigations are required to establish whether any of these connections are relevant to how the Bin1 gene acts in apoptosis and how its proapoptotic functions are controlled differentially from its functions in endocytosis, proliferation, and differentiation.

Modified dual signal model for c-Myc function

Two general models for how apoptosis is induced by c-Myc have been enunciated, the so-called conflict and dual signal models (Harrington *et al.*, 1994b; Packham and Cleveland, 1995). Figure 4 outlines each model. In the conflict model, apoptosis is proposed to be an indirect or distal response of the cell to an inappropriate growth signal generated by deregulated c-Myc. This model is a default and does not postulate a new function for c-Myc beyond that in cell division. In the dual signal model, Myc is proposed to induce apoptosis by directly regulating a death effector system(s). This system presumably includes downstream target genes that are death-specific but formally includes interactions with death effector signaling protein(s). The dual signal model postulates that c-Myc functions as regulator of cell death as well as cell division. Its strongest prediction is that at some levels the mitogenic and apoptotic properties of c-Myc should be distinct and hence separable. However, rudimentary structure-function analysis illustrates complete genetic overlap on c-Myc (Evan *et al.*, 1995), and as noted above, no functional divergence has occurred even in retroviral myc oncogenes, where selective pressures in evolution would be expected to produce rapid divergence if this were possible. Moreover, Max association and DNA binding is required for both apoptosis and proliferation (Amati *et al.*, 1993b). Thus, an inescapable conclusion from structural and biological evidence is that c-Myc coordinately primes cell death as it drives cell proliferation.

Although the functions of c-Myc can not be separated at the level of the protein, there is evidence that these functions can be separated on the basis of separate effector pathways. We propose that growth and death priming or sensitization functions are integrated in one pathway and that death triggering is mediated through a separate pathway (Figure 4, part 3 and Figure 5). The following lines of evidence support this modified dual signal model. First, it is possible in certain cases to arrest cells containing deregulated c-Myc without killing them (Gibson *et al.*, 1995; Packham and Cleveland, 1996; Ryan and Birnie, 1997). For example, c-Myc-expressing 32D cells undergo growth arrest in the absence of cell death when treated with non-hydrolyzable analogs of cAMP (Packham and Cleveland, 1996). IL3 withdrawal will still trigger cell death, indicating that IL3 and cAMP influence different signals that influence cell fate, consistent with a multiple effectors model (Packham and Cleveland, 1996). These observations are consistent with the demonstration that cAMP analogs will inhibit the ability of c-Myc to generate activators of apoptosis as measured in cell-free extracts (Ding *et al.*, 1998). A second line of support for dual signal is

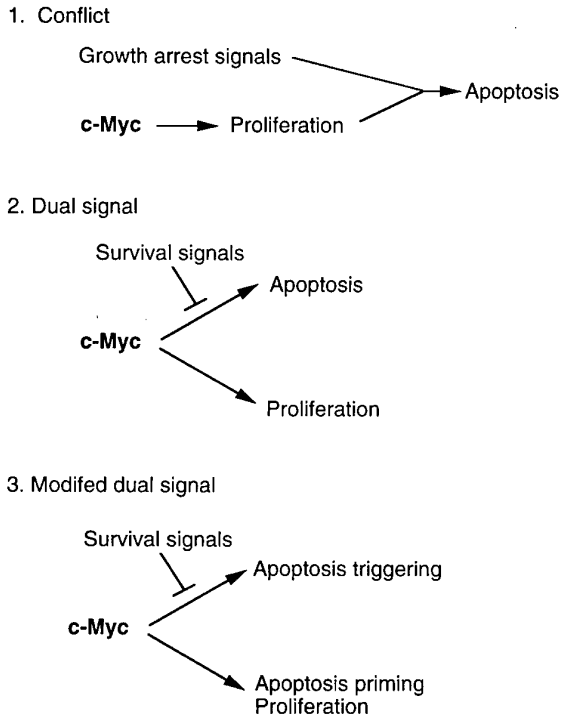


Figure 4 Conflict and dual signal models for Myc function. The models are adapted from Harrington and Evan (Harrington *et al.*, 1994b). In conflict, c-Myc functions solely in cell proliferation; apoptosis is the cellular response to an inappropriate signal from c-Myc when growth arrest signals are extant. In dual signal, c-Myc coordinately induces apoptosis and cell proliferation through different pathways; survival factors suppress the apoptosis pathway. In the modified dual signal model discussed in the text, c-Myc coordinately induces proliferation and primes apoptosis through one pathway and triggers apoptosis through a second pathway. In this model, survival factors suppress the apoptosis trigger pathway

provided by the identification of target genes which appear to be specifically involved in apoptosis. Activation of $p19^{ARF}$ by c-Myc, which is thought to be transcriptional (Dang, 1999), has been so implicated (Zindy *et al.*, 1998). Third, inhibition of CD95/Fas signaling blocks apoptosis but not proliferation by c-Myc in Rat1 fibroblasts (Hueber *et al.*, 1997). CD95/Fas ligand (CD95L/FasL) may be an additional target of c-Myc in certain cell types (Wang *et al.*, 1998a; D Sakamuro and GC Prendergast, unpublished observations). Lastly, interaction between c-Myc and Bin1 is necessary to activate apoptosis but dispensable to drive proliferation or malignant transformation, in both fibroblast and epithelial cell models (Sakamuro *et al.*, manuscript submitted). These results arguably offer the most direct support for the dual signal model insofar as they make a distinction in functions at the level of a binding protein.

Other reasons support the notion that the proapoptotic effector functions of c-Myc can be further discussed into 'death priming' and 'death triggering' steps. One reason is that protein synthesis inhibitors do not block c-Myc-mediated cell death (Evan *et al.*, 1992; Wagner *et al.*, 1994). This is telling because it is also clear that Max binding (Amati *et al.*, 1993b) and therefore transcriptional regulation of target genes by c-Myc is required for it to induce death (e.g. $p19^{ARF}$) (Dang, 1999). A second reason is inferred by the structure of Bin1, which while necessary for apoptosis by c-Myc does not have features of a transcription factor but rather features of a signal transduction protein (e.g. an SH3 domain). Bin1 may be linked to transcriptional programs, though, since it can inhibit the transactivation properties of c-Myc (Elliott *et al.*, 1999).

We hypothesize that c-Myc 'primes' death through transcriptional regulation of target genes, and 'triggers' death though some signaling mechanism that involves productive interactions between Bin1 and the 'primed' target gene products (see Figure 5). Priming would allow the Max requirement to be accommodated and triggering would provide a role for Bin1 consistent with its requirement and its structure as a signaling adaptor. If priming and triggering are separable, then c-Myc should still prime death in tumor cells where Bin1 is missing or inhibited. There is some support for this idea based on the ability of c-Myc to induce latent activators of caspase activity in cell-free extracts from tumor cells where Bin1 is lost (Fearnhead *et al.*, 1997, 1998; Ding *et al.*, 1998) and the ability of Bin1 to trigger death in such cells upon its reintroduction (K Elliott *et al.*, manuscript submitted). The triggering mechanism may have two parts, one that activates caspases and a second that serves to commit cells to a death decision. That these signals are separable is suggested by the ability of caspase inhibitors to block many classical features of apoptosis by c-Myc, but not the commitment to undergo cell death (McCarthy *et al.*, 1997). Examples of caspase-independent apoptosis associated with some role for the subnuclear domain termed ND10 (Ascoli and Maul, 1991) have appeared recently in studies of the leukemia breakpoint gene PML (Quignon *et al.*, 1998; Wang *et al.*, 1998b). Investigations of caspase-independent signals, possibly involving Bin1, may provide new insights into how cells commit to undergo apoptosis and how cell death is triggered.

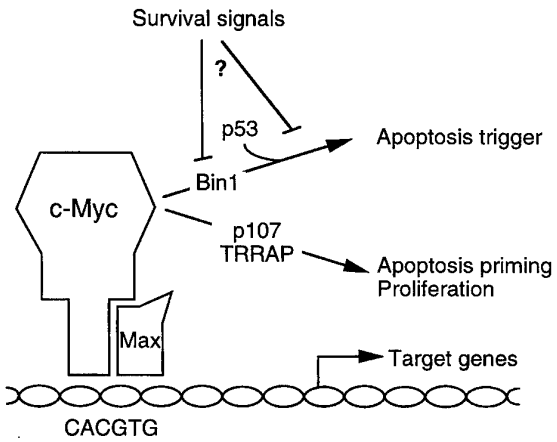


Figure 5 A modified dual signal model for c-Myc function. The model proposes that c-Myc activates proliferation and primes apoptosis through one pathway and triggers apoptosis through a second and mechanistically distinct pathway. The inability of protein synthesis inhibitors to block apoptosis in cells expressing deregulated c-Myc suggests that the apoptotic program is primed by gene regulation and triggered by a separate signaling process. In the model, death priming and proliferation involves regulation of specific target genes by c-Myc/Max complexes. The links between TRRAP and the transcription complex SAGA suggest it may be involved with the proliferation pathway. p107 interaction is proposed to co-ordinate gene regulation with the cell cycle. Max and gene regulation is required for proliferation and death priming but may be dispensable for death triggering. Conversely, Bin1 is dispensable for proliferation and death priming but necessary to trigger death once it is primed. p53 may contribute to sensitizing cells to the trigger, but is mechanistically distinct from Bin1 since the latter can mediate p53-independent death. Survival signals from cytokine and adhesion receptor pathways are proposed to target the triggering pathway for inactivation, possibly by targeting Bin1 itself since it is a phosphoprotein (Wechsler-Reya *et al.*, 1997a)

Summary and future directions

Recent advances strongly support a dual function model for c-Myc as a co-ordinate activator of cell proliferation and apoptosis. Both functions are intrinsic to c-Myc but evidence for specific death effector pathways is emerging. Such effector signals are hypothesized to include 'priming' and 'triggering' mechanisms associated with separable caspase-dependent and caspase-independent processes. Myc target genes implicated at some level include ODC, lactate dehydrogenase-A, $p19^{ARF}$, and possibly certain cell cycle regulators. The cytokines IGF-1 and PDGF regulate the apoptotic properties of c-Myc as do cell adhesion signals. The findings that c-Myc sensitizes the cells to death receptor signals from the TNF receptor and CD95/Fas, and may require signals from the latter receptor to kill cells, hint at regulatory links whose continued investigation may provide deeper insights into apoptosis by c-Myc and other oncogenes. Lastly, a specific role for the c-Myc-interacting cell fate adaptor Bin1 in apoptosis suggests the existence of a heretofore unrecognized nuclear-based death effector signaling pathway used by c-Myc.

While the new advances provide a stronger foundation for understanding the intrinsically dual nature of c-Myc function in growth and apoptosis, many pressing questions remain about how it kills cells. In particular, one would like to identify the molecular basis for the stochastic aspect of death. While sensitization of cells to apoptosis may 'lower the bar' to trigger death by minor insults (Evan and Littlewood, 1998), the mechanism by which cells reach the threshold to commit to engage the death program is still quite obscure. Caspase activation looks to be coordinated with but perhaps separable from the death commitment signal. To assess the significance of different caspases as well as components of the executioner machinery, death receptor DISCs, etc., it will be necessary to examine the effects of various null backgrounds on apoptosis by c-Myc or the effects on tumor development and apoptosis in crosses between various 'knockout' animals and *myc* transgenic mice. Pinpointing the death commitment signal may provide a powerful tool to address advanced cancer, since it would allow one to manipulate the threshold which must be crossed to commit to death. One question is whether there are cell cycle regulators or target genes with an unambiguous role in apoptosis. Another is whether the proapoptotic E2F member E2F1 has any role in mediating death by c-Myc, because of some evidence that c-Myc acts upstream of Rb and E2F in cell cycle regulation (Amati *et al.*, 1998). What is the exact relationship between c-Myc and p53 or between c-Myc and the TNF receptor or CD95/Fas? What are the targets of the antiapoptotic signals mediated by cytokines and matrix? These questions also impinge greatly on therapeutic possibilities in cancer. While c-Myc investigations have traditionally presented a minefield to the researcher, the pay-off for advances in understanding is likely to continue to be significant to our grasp of basic cell biological processes such as cell fate determination as well as to applied problems in cancer.

Acknowledgments

In preparing this review I relied in part upon comprehensive reviews cited in the text and apologize for the omission of many important original references. Critical reading from members of my laboratory, K Elliott, K Ge, D Sakamuro and M Summers, are gratefully appreciated. Our research is supported by grants from the NIH, the American Cancer Society, the US Army Breast and Prostate Research Programs, Merck and Co., Inc., The Mary Smith Foundation, and The Adler Foundation. GC Prendergast is a Pew Scholar in the Biomedical Sciences.

References

Adams JM and Cory S. (1992). *Cancer Surv.*, **15,** 119–141.
Adams JM and Cory S. (1998). *Science,* **281,** 1322–1326.
Alarcon RM, Rupnow BA, Graeber TG, Knox SJ and Giaccia AJ. (1996). *Cancer Res.*, **56,** 4315–4319.
Alexandrova N, Niklinski J, Bliskovsky V, Otterson GA, Blake M, Kaye FJ and Zajac-Kaye M. (1995). *Mol. Cell. Biol.*, **15,** 5188–5195.
Amati B, Alevizopoulos K and Vlach J. (1998). *Front. Biosci.*, **3,** D250–D258.
Amati B, Brooks MW, Levy N, Littlewood TD, Evan GI and Land H. (1993a). *Cell,* **72,** 233–245.
Amati B, Dalton S, Brooks MW, Littlewood TD, Evan GI and Land H. (1992). *Nature,* **359,** 423–426.
Amati B, Littlewood TD, Evan GI and Land H. (1993b). *EMBO J.*, **12,** 5083–5087.
Amundadottir, LT, Nass SJ, Berchem GJ, Johnson MD and Dickson RB. (1996). *Oncogene,* **13,** 757–765.
Armstrong DK, Isaacs JT, Ottaviano YL and Davidson NE. (1992). *Cancer Res.*, **52,** 3418–3424.
Asai A, Miyagi Y, Sugiyama A, Nagashima Y, Kanemitsu H, Obinata M, Mishima K and Kuchino Y. (1994). *Oncogene,* **9,** 2345–2352.
Ascoli CA and Maul GG. (1991). *J. Cell. Biol.*, **112,** 785–795.
Ashkenazi A and Dixit VM. (1998). *Science,* **281,** 1305–1308.
Askew DS, Ashmun RA, Simmons BC and Cleveland JL. (1991). *Oncogene,* **6,** 1915–1922.
Auvinen M, Passinen A, Andersson LC and Holtta E. (1992). *Nature,* **360,** 355–358.
Barone MV and Courtneidge SA. (1995). *Nature,* **378,** 509–512.
Baserga R, Resnicoff M and Dews M. (1997). *Endocrine,* **7,** 99–102.
Bauer F, Urdaci M, Aigle M and Crouzet M. (1993). *Mol. Cell. Biol.*, **13,** 5070–5084.
Beijersbergen RL, Hijmans EM, Zhu L and Bernards R. (1994). *EMBO J.*, **13,** 4080–4086.
Bello-Fernandez C, Packham G and Cleveland JL. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 7804–7808.
Bellosta P, Zhang Q, Goff SP and Basilico C. (1997). *Oncogene,* **15,** 2387–2397.
Ben-Yosef T, Yanuka O, Halle D and Benvenisty N. (1998). *Oncogene,* **17,** 165–171.
Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R and Weissberg P. (1998). *Science,* **282,** 290–293.
Bennett MR, Evan GI and Schwartz SM. (1995). *Circ. Res.*, **77,** 266–273.
Benvenisty N, Leder A, Kuo A and Leder P. (1993). *Genes and Dev.*, **6,** 2513–2523.
Berberich S, Hyde-deRuyscher N, Espenshade P and Cole M. (1992). *Oncogene,* **7,** 775–779.
Berberich SJ and Cold MD. (1992). *Genes Dev.*, **6,** 166–176.
Berns EM, Klijn JG, van PW, van SI, Portengen H and Foekens JA. (1992). *Cancer Res.*, **52,** 1107–1113.
Bissonnette RP, Echeverri F, Mahboubi A and Green DR. (1992). *Nature,* **359,** 552–554.
Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN and Weintraub H. (1990). *Science,* **250,** 1149–1152.
Blackwood E and Eisenman RN. (1991). *Science,* **251,** 1211–1217.
Blackwood E, Lüscher B and Eisenman RN. (1992). *Genes and Dev.*, **6,** 71–80.
Blagosklonny MV, Giannakakou P, el-Diery WS, Kingston DG, Higgs PI, Neckers L and Fojo T. (1997). *Cancer Res.*, **57,** 130–135.
Borg A, Baldetorp B, Ferno M, Olsson H and Sigurdsson H. (1992). *Int. J. Cancer,* **51,** 687–691.
Born T, Frost J, Schönthal A, Prendergast GC and Feramisco J. (1994). *Mol. Cell. Biol.*, **14,** 5741–5747.
Bouchard C, Staller P and Eilers M. (1998). *Trends Cell Biol.*, **8,** 202–206.
Bova GS and Isaacs WB. (1996). *World J. Urol.*, **14,** 338–346.
Boyd KE, Wells J, Gutman J, Bartley SM and Farnham PJ. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 13887–13892.

Brändström A, Westin P, Bergh A, Cajander S and Damber JE. (1994). *Cancer Res.*, **54,** 3594–3601.
Braun K, Holzl G, Pusch O and Hengstschlager M. (1998). *DNA Cell Biol.*, **17,** 789–798.
Breton AM and Aigle M. (1998). *Curr. Genet.*, **34,** 280–286.
Brizzio V, Gammie AE and Rose MD. (1998). *J. Cell. Biol.*, **141,** 567–584.
Brough DE, Hofmann TJ, Ellwood KB, Townley RA and Cole MD. (1995). *Mol. Cell. Biol.*, **15,** 1536–1544.
Bush A, Mateyak M, Dugan K, Obaya A, Adachi S, Sedivy J and Cole M. (1998). *Genes Dev.*, **12,** 3797–3802.
Butler MH, David C, Ochoa G-C, Freyberg Z, Daniell L, Grabs D, Cremona O and De Camilli P. (1997). *J. Cell. Biol.*, **137,** 1355–1367.
Cardone MH, Roy N, Stennicke HR, Salvessen GS, Franke TF, Standrbridge E, Frisch S and Reed JC. (1998). *Science,* **282,** 1318–1321.
Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA and Gruss P. (1998). *Cell,* **94,** 727–737.
Chang HY, Nishitoh H, Yang X, Ichijo H and Baltimore D. (1998). *Science,* **281,** 1860–1863.
Chao DT and Korsmeyer SJ. (1998). *Ann. Rev. Immunol.*, **16,** 395–419.
Chen G, Shi L, Litchfield DW and Greenberg AH. (1995). *J. Exp. Med.*, **181,** 2295–2300.
Cher ML, Bova GS, Moore DH, Small EJ, Carroll PR, Pin SS, Epstein JI, Isaacs WB and Jensen RH. (1996). *Cancer Res.*, **56,** 3091–3102.
Cherney BW, Bhatia K and Tosato G. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 12967–12971.
Chernova OB, Chernov MV, Ishizaka Y, Agarwal ML and Stark GR. (1998). *Mol. Cell. Biol.*, **18,** 536–545.
Chu K, Niu X and Williams LT. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 11894–11898.
Clark EA, King WG, Brugge JS, Symons M and Hynes RO. (1998). *J. Cell. Biol.*, **142,** 573–586.
Cole MD. (1986). *Ann. Rev. Genet.*, **20,** 361–384.
Crouch DH, Fincham VJ and Frame MC. (1996). *Oncogene,* **12,** 2689–2696.
Crouch DH, Fisher F, Clark W, Jayaraman PS, Goding CR and Gillespie DA. (1993). *Oncogene,* **8,** 1849–1855.
Crouzet M, Urdaci M, Dulau L and Aigle M. (1991). *Yeast,* **7,** 727–743.
Dang CV. (1999). *Mol. Cell. Biol.*, **19,** 1–11.
Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. (1997). *Cell,* **91,** 231–241.
David C, McPherson PS, Mundigl O and de Camilli P. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 331–335.
David C, Solimena M and De Camilli P. (1994). *FEBS Lett.*, **351,** 73–79.
de Stanchina E, McCurrach ME, Zindy F, Shieh SY, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ and Lowe SW. (1998). *Genes Dev.*, **12,** 2434–2442.
del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. (1997). *Science,* **278,** 687–689.
Dhanaraj SN, Marcus AM, Korah RM, Iwata K and Small MB. (1996). *Exp. Cell. Res.*, **224,** 52–62.
Ding HF, McGill G, Rowan S, Schmaltz C, Shimamura A and Fisher DE. (1998). *J. Biol. Chem.*, **273,** 28378–28383.
Dolnikov A, Ward RL, Hawkins NJ and Symonds G. (1996). *Oncogene,* **12,** 1189–1198.
Dong J, Naito M and Tsuruo T. (1997). *Oncogene,* **15,** 639–647.
Downward J. (1998). *Curr. Opin. Gene. Dev.*, **8,** 49–54.
Dropcho EJ. (1996). *Ann. Neurol.*, **39,** 659–667.
Eilers M, Picard D, Yamamoto KR and Bishop JM. (1989). *Nature,* **340,** 66–68.
Elliott K, Sakamuro D, Basu A, Du W, Wunner W, Staller P, Gaubatz S, Zhang H, Prochownik E, Eilers M and Prendergast GC. (1999). *Oncogene,* in press.
Ellis RE, Yuan JY and Horvitz HR. (1991). *Ann. Rev. Cell. Biol.*, **7,** 663–698.
Elson A, Deng C, Campos-Torres J, Donehower LA and Leder P. (1995). *Oncogene,* **11,** 181–190.
Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S. (1998). *Nature,* **391,** 43–50.
Eskes R, Antonsson B, Osen-Sand A, Montessuit S, Richter C, Sadoul R, Mazzei G, Nichols A and Martinou JC. (1998). *J. Cell. Biol.*, **143,** 217–224.
Evan GI, Brown L, Whyte M and Harrington E. (1995). *Curr. Biol.*, **7,** 825–834.
Evan GI and Littlewood TD. (1993). *Curr. Opin. Genet. Dev.*, **3,** 44–49.
Evan G and Littlewood TD. (1998). *Science,* **281,** 1317–1322.
Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ and Hancock DC. (1992). *Cell,* **69,** 119–128.
Facchini LM, Chen S and Penn LJ. (1994). *Cell Growth Diff.*, **5,** 637–646.
Facchini LM and Penn LZ. (1998). *FASEB J.*, **12,** 633–651.
Fanidi A, Harrington EA and Evan GI. (1992). *Nature,* **359,** 554–556.
Faris M, Latinis KM, Kempiak SJ, Koretzky GA and Nel A. (1998). *Mol. Cell. Biol.*, **18,** 5414–5424.
Fearnhead HO, McCurrach ME, O'Neill J, Zhang K, Lowe SW and Lazebnik YA. (1997). *Genes Dev.*, **11,** 1266–1276.
Fearnhead HO, Rodriguez J, Govek EE, Guo W, Kobayashi R, Hannon G and Lazebnik YA. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 13664–13669.
Ferre-D'Amare A, Prendergast GC, Ziff EB and Burley SK. (1993). *Nature,* **363,** 38–45.
Franke TF, Kaplan DR and Cantley LC. (1997). *Cell,* **88,** 435–437.
Frisch SM and Francis H. (1994). *J. Cell. Biol.*, **124,** 619–626.
Frisch SM and Ruoslahti E. (1997). *Curr. Opin. Cell. Biol.*, **9,** 701–706.
Galaktionov K, Chen X and Beach D. (1996). *Nature,* **382,** 511–517.
Gallant P, Shiio Y, Cheng PF, Parkhurst SM and Eisenman RN. (1996). *Science,* **274,** 1523–1527.
Gandarillas A and Watt FM. (1997). *Genes Dev.*, **11,** 2869–2882.
Gaubatz S, Meichle A and Eilers M. (1994). *Mol. Cell. Biol.*, **14,** 3853–3862.
Gibson AW, Cheng T and Johnston RN. (1995). *Exp. Cell. Res.*, **218,** 351–358.
Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW and Giaccia AJ. (1996). *Nature,* **379,** 88–91.
Grandori C and Eisenman RN. (1997). *Trends Biochem. Sci.*, **22,** 177–181.
Grant PA, Sterner DE, Duggan LJ, Workman JL and Berger SL. (1998). *Trends Cell Biol.*, **8,** 193–197.
Green DR. (1998). *Cell,* **94,** 695–698.
Green DR and Reed JC. (1998). *Science,* **281,** 1309–1312.
Gu W, Bhatia K, Magrath IT, Dang CV and Dalla-Favera R. (1994). *Science,* **264,** 251–254.
Gu W, Cechova K, Tassi V and Dalla-Favera R. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 2935–2939.
Guilherme A and Czech MP. (1998). *J. Biol. Chem.*, **273,** 33119–33122.
Guo Q, Xie J, Dang CV, Liu ET and Bishop JM. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 9172–9177.
Haas K, Staller P, Geisen C, Bartek J, Eilers M and Moroy T. (1997). *Oncogene,* **15,** 179–192.
Halazonetis TD and Kandil AN. (1991). *Proc. Natl. Acad. Sci. USA,* **88,** 6162–6166.
Hanson KD, Shichiri M, Follansbee MR and Sedivy JM. (1994). *Mol. Cell. Biol.*, **14,** 5748–5755.
Harrington E, Bennett MR, Fanidi A and Evan GI. (1994a). *EMBO J.*, **13,** 3286–3295.
Harrington EA, Fanidi A and Evan GI. (1994b). *Curr. Opin. Genet. Dev.*, **4,** 120–129.

He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B and Kinzler KW. (1998). *Science*, **281**, 1509–1512.
Hehir DJ, McGreal G, Kirwan WO, Kealy W and Brady MP. (1993). *J. Surg. Oncol.*, **54**, 207–209.
Heikkila R, Schwab G, Wickstrom E, Loke SL, Pluznik DH, Watt R and Neckers LM. (1987). *Nature*, **328**, 445–448.
Helbing CC, Veillette C, Riabowol K, Johnston RN and Garkavtsev I. (1997). *Cancer Res.*, **57**, 1255–1258.
Helbing CC, Wellington CL, Gogela-Spehar M, Cheng T, Pinchbeck GG and Johnston RN. (1998). *Onocgene*, **17**, 1491–1501.
Henriksson M and Lüscher B. (1996). *Adv. Cancer Res.*, **68**, 109–182.
Hermeking H and Eick D. (1994). *Science*, **265**, 2091–2093.
Hermeking H, Wolf DA, Kohlhuber F, Dickmanns A, Biollaud M, Fanning E and Eick D. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 10412–10416.
Hoang AT, Cohen KJ, Barrett JF, Bergstrom DA and Dang CV. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 6875–6879.
Hoang AT, Lutterbach B, Lewis BC, Yano T, Chou T-Y, Barrett JF, Raffeld M, Hann SR and Dang CV. (1995). *Mol. Cell. Biol.*, **15**, 4031–4042.
Holt JT, Redner RL and Nienhuis AW. (1988). *Mol. Cell. Biol.*, **8**, 963–973.
Hsu B, Marin MC, el-Naggar AK, Stephens LC, Brisbay S and McDonnell TJ. (1995). *Oncogene*, **11**, 175–179.
Hu Y, Ding L, Spencer DM and Nunez G. (1998). *J. Biol. Chem.*, **273**, 33489–33494.
Hueber AO, Zornig M, Lyon D, Suda T, Nagata S and Evan GI. (1997). *Science*, **278**, 1305–1309.
Huot J, Houle F, Rousseau S, Deschesnes RG, Shah GM and Landry J. (1998). *J. Cell. Biol.*, **143**, 1361–1373.
Inohara N, Gourley TS, Carrio R, Muniz M, Merino J, Garcia I, Koseki T, Hu Y, Chen S and Nunez G. (1998). *J. Biol. Chem.*, **273**, 32479–32486.
Inoue H, Tavoloni N and Hanafusa H. (1995). *Oncogene*, **11**, 231–238.
Janicke RU, Lee FH and Porter AG. (1994). *Mol. Cell. Biol.*, **14**, 5661–5670.
Janicke RU, Lin XY, Lee FH and Porter AG. (1996). *Mol. Cell. Biol.*, **16**, 5245–5253.
Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, Botz J, Wessbecher J, Draetta G and Eilers M. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 3685–3689.
Jenkins RB, Qian J, Lieber MM and Bostwick DG. (1997). *Cancer Res.*, **57**, 524–531.
Jiang MC, Yang-Yen HF, Lin JK and Yen JJ. (1996). *Oncogene*, **13**, 609–616.
Juo P, Kuo CJ, Yuan J and Blenis J. (1998). *Curr. Biol.*, **8**, 1001–1008.
Kaddurah-Daouk R, Papoulas O, Greene JM, Baldwin AS and Kingston RE. (1987). *Genes Dev.*, **1**, 347–357.
Kadlec L and Pendergast A-M. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 12390–12395.
Kagaya S, Kitanaka C, Noguchi K, Mochizuki T, Sugiyama A, Asai A, Yasuhara N, Eguchi Y, Tsujimoto Y and Kuchino Y. (1997). *Mol. Cell. Biol.*, **17**, 6736–6745.
Kang Y, Cortina R and Perry RR. (1996). *J. Natl. Cancer Inst.*, **88**, 279–284.
Kangas A, Nicholson DW and Hottla E. (1998). *Oncogene*, **16**, 387–398.
Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A and Green DR. (1998). *Mol. Cell.*, **1**, 543–551.
Kasibhatla S, Genestier L and Green DR. (1999). *J. Biol. Chem.*, **274**, 987–992.
Kato G, Lee WMF, Chen L and Dang C. (1992). *Genes Dev.*, **6**, 81–92.
Kato GJ, Barrett J, Villa-Garcia M and Dang CV. (1990). *Mol. Cell. Biol.*, **10**, 5914–5920.
Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J and Evan G. (1997). *Nature*, **385**, 544–548.
Kelekar A and Thompson CB. (1998). *Trends Cell Biol.*, **8**, 324–330.
Kelly K and Siebenlist U. (1986). *Ann. Rev. Immunol.*, **4**, 317–338.
Kerkhoff E, Bister K and Klempnauer K-H. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 4323–4327.
Kerr JF, Wyllie AH and Currie AR. (1972). *Br. J. Cancer*, **26**, 239–257.
Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne PH and Downward J. (1997). *EMBO J.*, **16**, 2783–2793.
King WG, Mattaliano MD, Chan TO, Tsichlis PN and Brugge JS. (1997). *Mol. Cell. Biol.*, **17**, 4406–4418.
Klefstrom J, Arighi E, Littlewood T, Jaattela M, Saksela E, Evan GI and Alitalo K. (1997). *EMBO J.*, **16**, 7382–7392.
Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M and Alitalo K. (1994). *EMBO J.*, **13**, 5442–5450.
Koumenis C and Giaccia A. (1997). *Mol. Cell. Biol.*, **17**, 7306–7316.
Kreipe H, Feist H, Fischer L, Felgner J, Heidorn K, Mettler L and Parwaresch R. (1993). *Cancer Res.*, **53**, 1956–1961.
Kretzner L, Blackwood EM and Eisenman RN. (1992a). *Nature*, **359**, 426–429.
Kretzner L, Blackwood EM and Eisenman RN. (1992b). *Curr. Top. Microbiol. Immunol.*, **182**, 435–443.
Kumar CC. (1998). *Oncogene*, **17**, 1365–1373.
Kyprianou N, English HF, Davidson NE and Isaacs JT. (1991). *Cancer Res.*, **51**, 162–166.
Kyprianou N, English HF and Isaacs JT. (1990). *Cancer Res.*, **50**, 3748–3753.
Lanoix J, D'Agati V, Szabolcs M and Trudel M. (1996). *Oncogene*, **13**, 1153–1160.
Laster SM and Mackenzie JMJ. (1996). *Microsc. Res. Tech.*, **34**, 272–280.
Lebowitz PF, Davide JP and Prendergast GC. (1995). *Mol. Cell. Biol.*, **15**, 6613–6622.
Lee LA, Dolde C, Barrett J, Wu CS and Dang CV. (1996). *J. Clin. Invest.*, **97**, 1687–1695.
Lee YJ, Galoforo SS, Berns CM, Tong WP, Kim HR and Corry PM. (1997). *J. Cell. Sci.*, **110**, 681–686.
Lemaitre JM, Buckle RS and Mechali M. (1996). *Adv. Cancer Res.*, **70**, 95–144.
Lenahan MK and Ozer HL. (1996). *Oncogene*, **12**, 1847–1854.
Levine AJ. (1993). *Ann. Rev. Biochem.*, **62**, 623–651.
Lewis JM, Baskaran R, Taagepera S, Schwartz MA and Wang JY. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 15174–15179.
Li H, Zhu H, Xu CJ and Yuan J. (1998). *Cell*, **94**, 491–501.
Li L, Nerlov C, Prendergast G, MacGregor D and Ziff EB. (1994). *EMBO J.*, **13**, 4070–4079.
Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X. (1997). *Cell*, **91**, 479–489.
Li WX, Chen CH, Ling CC and Li GC. (1996). *Radiat. Rad.*, **145**, 324–330.
Liu AX, Testa JR, Hamiltion TC, Jove R, Nicosia SV and Cheng JQ., (1998). *Cancer Res.*, **58**, 2973–2977.
Liu X, Zou H, Slaughter C and Wang X. (1997). *Cell*, **89**, 175–184.
Lotem J and Sachs L. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 9672–9676.
Luo X, Budihardjo I, Zou H, Slaughter C and Wang X. (1998). *Cell*, **94**, 481–490.
Lutz W, Fulda S, Jeremias I, Debatin KM and Schwab M. (1998). *Oncogene*, **17**, 339–346.
Ma A, Moroy T, Collum R, Weintraub H, Alt FW and Blackwell TK. (1993). *Oncogene*, **8**, 1093–1098.
Mäkelä TP, Koskinen PJ, Västrik I and Alitalo K. (1992). *Science*, **256**, 373–377.

Mao NC, Steingrimsson EJD, Ruiz J, Wasserman W, Copeland NG, Jenkins NA and Prendergast GC. (1999). *Genomics,* in press.
Marhin WW, Chen S, Facchini LM, Fornace AJJ and Penn LZ. (1997). *Oncogene,* **14,** 2825–2834.
Martin SJ and Green DR. (1995). *Cell,* **82,** 349–352.
Mateyak MK, Obaya AJ, Adachi S and Sedivy JM. (1997). *Cell Growth Diff.,* **8,** 1039–1048.
Mayo MW, Wang CY, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ and Baldwin AS. (1997). *Science,* **278,** 1812–1815.
McCarthy NJ, Whyte MKB, Gilbert CS and Evan GI. (1997). *J. Cell. Biol.,* **136,** 215–227.
McDonnell TJ, Troncoso P, Brisbay SM, Logothetis C, Chung LW, Hsieh JT, Tu SM and Campbell ML. (1992). *Cancer Res.,* **52,** 6940–6944.
McGill G, Shimamura A, Bates RC, Savage RE and Fisher DE. (1997). *J. Cell. Biol.,* **138,** 901–911.
McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD and Cole MD. (1998). *Cell,* **94,** 363–374.
Metzstein MM, Stanfield GM and Horvitz HR. (1998). *Trends Genet.,* **14,** 410–416.
Milner AE, Grand RJ, Waters CM and Gregory CD. (1993). *Oncogene,* **8,** 3385–3391.
Miltenberger RJ, Sukow KA and Farnham PJ. (1995). *Mol. Cell. Biol.,* **15,** 2527–2535.
Mittelstadt PR and Ashwell JD. (1998). *Mol. Cell. Biol.,* **18,** 3744–3751.
Miyashita T, Krajewski S, Krajewska M, Wang H-G, Lin HK, Liebermann DA, Hoffmann B and Reed JC. (1994). *Oncogene,* **9,** 1799–1805.
Miyashita T and Reed JC. (1995). *Cell,* **80,** 293–299.
Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R and Fassler R. (1997). *Genes Dev.,* **11,** 1938–1948.
Moshier JA, Dosescu J, Skunca M and Luk GD. (1993). *Cancer Res.,* **53,** 2618–2622.
Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN and Rosen N. (1998). *J. Biol. Chem.,* **273,** 29864–29872.
Mukherjee B, Morgenbesser SD and De PR. (1992). *Genes Dev.,* **6,** 1480–1492.
Munn AL, Stevenson BJ, Geli MI and Riezman H. (1995). *Mol. Biol. Cell.,* **6,** 1721–1742.
Natoli G, Costanzo A, Ianni A, Templeton DJ, Woodgett JR, Balsano C and Levero M. (1997). *Science,* **275,** 200–203.
Nesbit CE, Grove LE, Yin X and Prochownik EV. (1998). *Cell Growth Diff.,* **9,** 731–741.
Nishitoh H, Saitoh M, Mochida Y, Takeda K, Nakano H, Rothe M, Miyazono K and Ichijo H. (1998). *Mol. Cell.,* **2,** 389–395.
O'Connor R, Kauffmann-Zeh A, Liu Y, Lehar S, Evan GI, Baserga R and Blattler WA. (1997). *Mol. Cell. Biol.,* **17,** 427–435.
Okura T, Gong L, Kamitani T, Wada T, Okura I, Wei CF, Chang HM and Yeh ET. (1997). *J. Immunol.,* **157,** 4277–4281.
Owen DJ, Wigge P, Vallis Y, Moore JD, Evans PR and McMahon HT. (1998). *EMBO J.,* **17,** 5273–5285.
Packham G and Cleveland JL. (1994). *Mol. Cell. Biol.,* **14,** 5741–5747.
Packham G and Cleveland JL. (1995). *Biochim. Biophys. Acta,* **1242,** 11–28.
Packham G and Cleveland JL. (1996). *Oncogene,* **13,** 461–469.
Packham G and Cleveland JL. (1997). *Oncogene,* **15,** 1219–1232.
Pan G, O'Rourke K and Dixit VM. (1998). *J. Biol. Chem.,* **273,** 5841–5845.
Parsons JT and Parsons SJ. (1997). *Curr. Opin. Cell. Biol.,* **9,** 187–192.
Peter ME, Heufelder AE and Hengartner MO. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 12736–12737.
Petropoulos C, Givol I and Hughes SH. (1996). *Oncogene,* **12,** 2611–2621.
Philipp A, Schneider A, Väsrik I, Finke K, Xiong Y, Beach D, Alitalo K and Eilers M. (1994). *Mol. Cell. Biol.,* **14,** 4032–4043.
Prendergast GC. (1997). Myc structure and function. In: *Oncogenes as Transcriptional Regulators.* Birkhauser Verlag: Boston. pp. 1–28.
Prendergast GC, Hopewell R, Gorham B and Ziff EB. (1992). *Genes Dev.,* **6,** 2429–2439.
Prendergast GC, Lawe D and Ziff EB. (1991). *Cell,* **65,** 395–407.
Prendergast GC and Ziff EB. (1991). *Science,* **251,** 186–189.
Prochownik EV and Van Antwerp ME. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 960–964.
Quignon F, De Bels F, Koken M, Feunteun J, Ameisen JC and de The H. (1998). *Nat. Genet.,* **20,** 259–265.
Raff MC. (1992). *Nature,* **356,** 397–400.
Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS and Buttyan R. (1995). *Cancer Res.,* **55,** 4438–4445.
Ramjaun AR and McPherson PS. (1998). *J. Neurochem.,* **70,** 2369–2376.
Ramjaun AR, Micheva KD, Bouchelet I and McPherson PS, (1997). *J. Biol. Chem.,* **272,** 16700–16706.
Reddy CD, Dasgupta P, Saikumar P, Dudek H, Rauscher FJ and Reddy EP. (1992). *Oncogene,* **7,** 2085–2092.
Reed JC, Jurgensmeier JM and Matsuyama S. (1998). *Biochim. Biophys. Acta,* **1366,** 127–137.
Reinhard C, Shamoon B, Shyamala V and Williams LT. (1997). *EMBO J.,* **16,** 1080–1092.
Reisman D, Elkind NB, Roy B, Beamon J and Rotter V. (1993). *Cell Growth Diff.,* **4,** 57–65.
Rohn JL, Hueber AO, McCarthy NJ, Lyon D, Navarro P, Burgering BM and Evan GI. (1998). *Oncogene,* **17,** 2811–2818.
Roy AL, Malik S, Meisterernst M and Roeder RG. (1993). *Nature,* **365,** 355–361.
Rudolph B, Saffrich R, Zwicker J, Henglein B, Muller R, Ansorge W and Eilers M. (1996). *EMBO J.,* **15,** 3065–3076.
Rudolph C, Halle JP and Adam G. (1998). *Exp. Cell. Res.,* **239,** 361–369.
Ruggeri BA, Huang LMW, Cheng JQ and Testa JR. (1998). *Mol. Carcin.,* **21,** 81–86.
Rupnow BA, Murtha AD, Alarcon RM, Giaccia AJ and Knox SJ. (1998). *Cancer Res.,* **58,** 1779–1784.
Ryan KM and Birnie GD. (1996). *Biochem. J.,* **314,** 713–721.
Ryan KM and Birnie GD. (1997). *Oncogene,* 2835–2843.
Sakamuro D, Elliott K, Wechsler-Reya R and Prendergast GC. (1996). *Nature Genet.,* **14,** 69–77.
Sakamuro D, Eviner V, Elliott K, Showe L, White E and Prendergast GC. (1995). *Oncogene,* **11,** 2411–2418.
Sakamuro D, Sabbatini P, White E and Prendergast GC. (1997). *Oncogene,* **15,** 887–898.
Saleh A, Schieltz D, Ting N, McMahon SB, Litchfield DW, Yates JRR, Lees-Miller SP, Cole MD and Brandl CJ. (1998). *J. Biol. Chem.,* **273,** 26559–26565.
Sawyers CL, Callahan W and Witte ON. (1992). *Cell,* **70,** 901–910.
Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME. (1998). *EMBO J.,* **17,** 1675–1687.
Scheid MP and Duronio V. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 7439–7444.
Schreiber-Agus N, Stein D, Chen K, Goltz JS, Stevens L and DePinho RA. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 1235–1240.

Selvakumaran M, Lin H-K, Miyashita T, Wang H-G, Krajewski S, Reed JC, Hoffman B and Liebermann D. (1994a). *Oncogene*, **9**, 1791–1799.

Selvakumaran M, Lin HK, Sjin RT, Reed JC, Liebermann DA and Hoffman B. (1994b). *Mol. Cell. Biol.*, **14**, 2352–2360.

Shantz LM and Pegg AE. (1994). *Cancer Res.*, **54**, 2313–2316.

Sherr CJ. (1998). *Genes Dev.*, **12**, 2984–2991.

Shi L, Nishioka WK, Thng J, Bradbury EM, Litchfield DW and Greenberg AH. (1994). *Science*, **263**, 1143–1145.

Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP and Green DR. (1992). *Science*, **257**, 212–214.

Shim H, Chun YS, Lewis BC and Dang CV. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 1511–1516.

Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R and Dang CV. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 6658–6663.

Shiu RP, Watson PH and Dubik D. (1993). *Clin. Chem.*, **39**, 353–355.

Sivadon P, Bauer F, Aigle M and Crouzet M. (1995). *Mol. Gen. Genet.*, **246**, 485–495.

Sivadon P, Peypouquet MF, Doignon F, Aigle M and Crouzet M. (1997). *Yeast*, **13**, 747–761.

Sklar MD, Thompson E, Welsh MJ, Liebert M, Harney J, Grossman HB, Smith M and Prochownik EV. (1991). *Mol. Cell. Biol.*, **11**, 3699–3710.

Sonenshein GE. (1997). *J. Immunol.*, **158**, 1994–1997.

Spencer CA and Groudine M. (1991). *Adv. Cancer Res.*, **56**, 1–48.

Stone J, de Lange T, Ramsay G, Jakobovits E, Bishop JM, Varmus H and Lee W. (1987). *Mol. Cell. Biol.*, **7**, 1697–1709.

Strasser A, Harris AW, Bath ML and Cory S. (1990). *Nature*, **348**, 331–333.

Strohmeyer TG and Slamon DJ. (1994). *J. Urol.*, **151**, 1479–1497.

Suen T-C and Hung M-C. (1991). *Mol. Cell. Biol.*, **11**, 354–362.

Taagepera S, McDonald D, Loeb JE, Whitaker LL, McElroy AK, Wang JY and Hope TJ. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 7457–7462.

Taira T, Maeda J, Onishi T, Kitaura H, Yoshida S, Kato H, Ikeda M, Tamai K, Iguchi-Ariga SM and Ariga H. (1998). *Genes Cells*, **3**, 549–565.

Thornberry NA and Lazebnik Y. (1998). *Science*, **281**, 1312–1316.

Tikhonenko AT, Black DJ and Linial ML. (1997). *J. Biol. Chem.*, **271**, 30741–30747.

Tobias KE, Shor J and Kahana C. (1995). *Oncogene*, **11**, 1721–1727.

Troppmair J, Cleveland JL, Askew DS and Rapp UR. (1992). *Curr. Top. Microbiol. Immunol.*, **182**, 453–460.

Trudel M, Lanoix J, Barisoni L, Blouin MJ, Desforges M, L'Italien C and D'Agati V. (1997). *J. Exp. Med.*, **186**, 1873–1884.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC and Croce CM. (1984). *Science*, **226**, 1097–1099.

Tsutsui K, Maeda Y, Tsutsui K, Seki S and Tokunaga A. (1997). *Biochem. Biophys. Res. Comm.*, **236**, 178–183.

Ueda K and Ganem D. (1996). *J. Virol.*, **70**, 1375–1383.

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T *et al.* (1998). *Immunity*, **9**, 267–276.

Vaux DL, Cory S and Adams JM. (1988). *Nature*, **335**, 440–442.

Wagner AJ, Kokonitis JM and Hay N. (1994). *Genes Dev.*, **8**, 2817–2830.

Wagner AJ, Meyers C, Laimins LA and Hay N. (1993a). *Cell Growth Diff.*, **4**, 879–883.

Wagner AJ, Small MB and Hay N. (1993b). *Mol. Cell. Biol.*, **13**, 2432–2440.

Wajant H, Johannes FJ, Haas E, Siemienski K, Schwenzer R, Schubert G, Weiss T, Grell M and Scheurich P. (1998). *Curr. Biol.*, **8**, 113–116.

Wang R, Brunner T, Zhang L and Shi Y. (1998a). *Oncogene*, **17**, 1503–1508.

Wang W, Wykrzykowska J, Johnson T, Sen R and Sen J. (1999). *J. Immunol.*, **162**, 314–322.

Wang Y, Ramquivst T, Szekely L, Axelson H, Klein G and Wiman KG. (1993a). *Cell Growth Diff.*, **4**, 467–473.

Wang Y, Szekely L, Okan I, Klein G and Wiman KG. (1993b). *Onocogene*, **8**, 3427–3431.

Wang ZG, Ruggero D, Ronchetti S, Zhong S, Gaboli M, Rivi R and Pandolfi PP. (1998b). *Nat. Genet.*, **20**, 266–272.

Warburg O. (1956). *Science*, **123**, 309–314.

Watson PH, Safneck JR, Le K, Dubik D and Shiu RP. (1993). *J. Natl. Cancer Inst.*, **85**, 902–907.

Wechsler-Reya R, Elliott K, Herlyn M and Prendergast GC. (1997a). *Cancer Res.*, **57**, 3258–3263.

Wechsler-Reya R, Elliott K and Prendergast GC. (1998). *Mol. Cell. Biol.*, **18**, 566–575.

Wechsler-Reya R, Sakamuro D, Zhang J, Duhadaway J and Prendergast GC. (1997b). *J. Biol. Chem.*, **272**, 31453–31458.

Wenzel A, Cziepluch C, Hamann U, Schürmann J and Schwab M. (1991). *EMBO J.*, **10**, 3703–3712.

Wigge P, Vallis Y and McMahon HT. (1997). *Curr. Biol.*, **7**, 554–560.

Williams GT. (1991). *Cell*, **65**, 1097–1098.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW and Mak TW. (1998). *Genes Dev.*, **12**, 806–819.

Wu M, Arsura M, Bellas RE, FitzGerald MJ, Lee H, Schauer SL, Sherr DH and Sonenshein GE. (1996a). *Mol. Cell. Biol.*, **16**, 5015–5025.

Wu S, Pena A, Korcz A, Soprano DR and Soprano KJ. (1996b). *Oncogene*, **12**, 621–629.

Wu X, Senechal K, Neshat MS, Whang YE and Sawyers CL. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 15587–15591.

Wyllie AH. (1993). *Br. J. Cancer*, **67**, 205–208.

Wyllie AH, Rose KA, Morris RG, Steel CM, Foster E and Spandidos DA. (1987). *Br. J. Cancer*, **56**, 251–259.

Xiao Q, Claassen G, Shi J, Adachi S, Sedivy J and Hann SR. (1998). *Genes Dev.*, **12**, 3803–3808.

Xu Y, Nguyen Q, Lo DC and Czaja MJ. (1997). *J. Cell. Physiol.*, **170**, 192–199.

Yang B-S, Geddes TJ, Pogulis RJ, de Crombrugghe B and Freytag SO. (1991). *Mol. Cell. Biol.*, **11**, 2291–2295.

Yang B-S, Gilbert JD and Freytag SO. (1993). *Mol. Cell. Biol.*, **13**, 3093–3102.

Yang X, Khosravi-Far R, Chang HY and Baltimore D. (1997). *Cell*, **89**, 1067–1076.

Yao KS, Clayton M and O'Dwyer PJ. (1995). *J. Natl. Cancer Inst.*, **87**, 117–122.

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV and Mak TW. (1998). *Science*, **279**, 1954–1958.

Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, de la Pompa JL, Ferrick D, Hum B, Iscove N, Ohashi P, Rothe M, Goeddel DV and Mak TW. (1997). *Immunity*, **7**, 715–725.

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM and Mak TW. (1998). *Cell*, **94**, 739–750.

Yu K, Ravera CP, Chen YP and McMahon G. (1997). *Cell Growth Diff.*, **8**, 731–742.

Yuan J. (1996). *J. Cell. Biochem.*, **60**, 4–11.

Zhan Y, Cleveland JL and Stevens JL. (1997). *Mol. Cell. Biol.*, **17,** 6755–6764.
Zhang H, Fan S and Prochownik EV. (1997). *J. Biol. Chem.*, **272,** 17416–17424.
Zhang J, Cado D, Chen A, Kabra NH and Wilson A. (1998). *Nature,* **392,** 296–300.
Zheng B and Clemmons DR. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 11217–11222.
Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ and Roussel MF. (1998). *Genes Dev.*, **12,** 2424–2433.
Zornig M, Busch G, Beneke R, Gulbins E, Lang F, Ma A, Korsmeyer S and Moroy T. (1995). *Oncogene,* **11,** 2165–2174.
Zornig M, Hueber AO and Evan G. (1998). *Curr. Biol.*, **8,** 467–470.
Zou H, Henzel WJ, Liu X, Lutschg A and Wang X. (1997). *Cell,* **90,** 405–413.